Transcription and RNA processing of the $tRNA_{glu}$- *cob* genome segment of yeast mitochondrial DNA

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I. Introduction

Mitochondria are rather variable, double membrane bound particles which have been shown to be the sites of gas exchange within the cell. They are the location of a sequence of reactions (known as the tricarboxylic acid cycle or Krebs' cycle) in which pyruvate, largely produced as a result of glycolysis, is oxidised to carbon dioxide and water. This oxidation of pyruvate is mediated by the production of reduced coenzymes which form the start of a catena of oxidations and reductions, terminating in the reduction of molecular oxygen to water. The catena is known as the respiratory chain and at specific points along this chain ATP is produced from ADP in a process termed oxidative phosphorylation. Mitochondria are thus the sites of cellular respiration, which may be defined as the process in which organic substrates are oxidised to yield carbon dioxide and energy with the accompanying reduction of molecular oxygen to water.

Structure

Mitochondria when observed by phase contrast microscopy, appear to be in continuous movement, to change shape rapidly and also to divide. These changes make precise estimates of both size and number difficult. However, on average, mitochondria appear as cylinders about 3-5 um long and 0.5-1.0 um in diameter. They vary in number from about 20 to 10^5 per cell; in maize root cap the numbers per cell (100- 3000) are roughly similar to those present in rat liver (500-1400).

Mitochondria are of particular interest because they contain their own genome. Relatively few proteins are encoded by mitochondrial DNA (mtDNA) and most of the proteins found in mitochondria are encoded by nuclear DNA, made on cytosolic ribosomes and then imported into the organelle.

Genome size and capacity

Mitochondria show a vast range of genome size, from the compact 16 kb of human mitochondria (Anderson et al., 1981), the 75 kb of yeast (Sanders et al., 1977), through to the 2700 kb of cucurbitaceae mitochondria (Ward et al., 1981).Genes may be tightly packed and even overlapping, as in mammalian mtDNA (Anderson et al., 1981), or spread out and interlaced with introns, as in yeast (Haid et al., 1979, Van Ommen et al., 1979) and the filamentous fungi (Burger, 1984, Davies, 1982). There is apparently no preferred gene order or obvious functional clustering of genes, although order tends to be conserved over short evolutionary distances.

Mitochondrial biogenesis

Mitochondrial biogenesis involves an intricate interplay between the nuclear and the mitochondrial genome. Mitochondrial genomes of many organisms have undergone extensive genetic and biochemical investigation. They appear to be extremely limited in their coding capacity. All mitochondrial genomes encode the RNA molecules involved in the mitochondrial protein synthetic machinery (Van Ommen, et al., 1979), namely the two rRNAs three in the case of plants (Leaver et al., 1984) and a complete set

of tRNAs. These genes allow the translation of a small number of genes coding for respiratory enzymes of the mitochondrial inner membrane: cytochrome oxidase I, II and III, cytochrome b and depending on the organism two or three units of the ATPase synthetase complex (Table I and Grivell 1983 for review). The precise number of mitochondrial genes can vary from one organism to another; for example, a gene coding for a mitochondrial ribosome associated protein is present in fungal mitochondria but absent in human mitochondria (Terpstra et al., 1979, La Polla et al., 1982).

Polypeptide	gene
Cytochrome c oxidase:	
Subunit I	oxi3
Subunit II	oxi1
Subunit III	oxi2
Apocytochrome b	cob
ATPase complex:	
Subunit 6	oli2
Subunit 8	aap1
Subunit 9	oli1
Ribosome-associated protein	var1

Table I: Yeast mitochondrially encoded components of the respiratory pathway.

Translation and codon usage

Translation of mature mRNAs also requires both nuclear and mitochondrial gene products. Mitochondrial ribosomes are themselves a mosaic of mitochondrial gene products (the RNAs and, at least in fungi, one protein) and of nuclear gene

products synthesised on cytoplasmic ribosomes (the bulk of the mitochondrial proteins). Whilst the tRNAs are coded for by the mitochondrial DNA the activating enzymes are of nuclear origin (Schneller et al., 1978). Furthermore, these tRNA activating enzymes appear to be shared by the cytosol and mitochondria (Hopper et al., 1982).

Mitochondria use their own variations on the genetic code. The most striking deviation from the once 'universal' version is the use of UGA to specify tryptophan and this is found in all mitochondrial genetic systems studied so far. Besides this, however, many organisms use additional, non standard codon assignments. It is sometimes assumed that these unusual assignments are relics of the primitive origin of the mitochondrial genetic system. Infact, quite the opposite seems to be true: mammals decode AGA(G) codons as stop (Anderson et al., 1981) while insects decode it as serine(De Bruijn, 1983), and this difference must reflect a change that has occured since their divergence 680 million years ago. Similarly, AUA codes for methionine in yeast (Hudspeth et al., 1982), insects (De Bruijn et al., 1983) and mammals (Barrell et al., 1979), but for isoleucine in all other organisms. The fact that within the fungi the AUA - methionine assignment is restricted to yeast argues strongly for a relatively late fixation in evolution.

Transcription and RNA processing

In human mitochondria (and probably in other mammals too) both DNA strands are completely transcribed from sites near the

origin of H-strand replication (Murphy et al., 1975). Mature gene-sized RNAs are then generated from the nascent transcripts by cleavage at the boundaries of the tRNA sequences that flank virtually every gene (Ojala et al., 1981). Nevertheless, levels of the two rRNAs are 30-60 fold higher than other transcripts. This is accounted for by the presence of two overlapping transcription processes. Initiation of RNA synthesis at a site just upstream of the $tRNA_{phe}$ gene occurs frequently and gives rise to transcripts terminating at the 3' end of the 16S rRNA. Initiation at the second site, which is located downstream of the first , near the 5' end of the small rRNA, is much less frequent and results in the synthesis of a polycistronic RNA corresponding to almost the entire H-strand. An implicit consequence of this situation must be that the fate of each transcript is determined from the start by either the involvement of two different RNA polymerases or the extra sequences present at the 5' end (Attardi, 1984).

In yeast scope for differential transcription control is far more extensive. Some 19 different sites of transcriptional initiation have been identified (Christianson et al., 1983), 13 of these have been precisely mapped in sequenced regions of the genome, either upstream of known genes or in putative origins of DNA replication (ori or rep sequences). Initiation of RNA synthesis occurs in all cases within a short conserved sequence which is either ATATAAGTA or a close variant of it (Osinga et al., 1982, Edwards et al., 1983, Tabak et al., 1983). Several genes lack this nonanucleotide and available evidence suggests that these are not transcribed independently. They form part of larger transcriptional units initiated far upstream and their

mature RNAs are subsequently generated by processing. The genes transcribed in this way form a rather haphazard collection so that the possible advantages of this organisation are not immediately apparent.

Although the transcription of mitochondrial genes appears to be carried out solely by nuclearly encoded factors, mtRNA processing requires both nuclearly and, at least in fungi, mitochondrially encoded products. In yeast, for example, the splicing of introns from the primary transcript of split genes needs the presence of intron encoded maturases (Lazowska et al, 1980, Hanson et al., 1982). But at the same time, intron splicing also requires functional nuclear gene products (Pillar et al., 1983, Dieckman et al., 1982, Faye and Simon, 1983). In addition, a specific mitochondrial transcript is needed, also in yeast, for the maturation of tRNAs (Miller et al., 1984). Three other major functions for the biogenesis of mitochondria seem to involve solely nuclear encoded products: the import of cytoplasmically made polypeptides into mitochondria, the replication and the transcription of mtDNA.

Mitochondrial mutants

S. cerivisiae is a facultative anaerobe which allows the isolation of mutants of the respiratory system. Yeast mitochondrial mutants exist in two forms: rho^- mutants occur spontaneously at high frequency and are the result of large deletions of the mtDNA, whereby the retained segment is regularly amplified around the rho^- genome (see figure 1.).

The rho⁻ genome may be transcribed but the genes it contains are never expressed into proteins because essential genes for mitochondrial protein synthesis are deleted. Rho⁻ mutants offer a virtually unlimited source of various deletions which have been essential in the construction of the genetic map in this species. In addition, each rho⁻ mutant provides a source of purified mtDNA fragments, extremely useful for both molecular and genetic studies on recombination, gene expression or gene amplification.

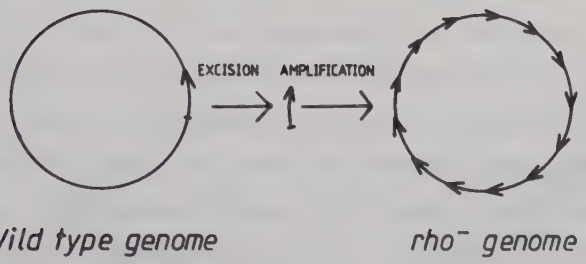

Figure 1. Diagramatic representation of the process of yeast mitochondrial rho⁻ genome formation which involves consecutive excision and amplification of the retained segment. This diagram shows the formation of a tandemly reiterated genome (head-to-tail). Repeat units can also be arranged as palindromes (head-to-head, tail-to-tail).

Mitochondrial mutations of S.cerivisiae are also detected in the form of point mutations or small alterations of mtDNA (mit⁻), affecting a specific gene or function. A multitude of respiration deficient mutants resulting from point mutations have now been mapped in the mitochondrial genes encoding:

apocytochrome b (the *cob* gene), the cytochrome oxidase subunits I, II and III (genes *oxi3* , *oxi1* and *oxi2* respectively), subunits 9, 6 and 8 of ATPase (genes *oli1* , *oli2* and *aap1*) respectively. In the case of the *cob* gene and the *oxi3* gene which are mosaic, mit^- mutations have also been found within the introns. Other point mutations which map at a few specific loci within the previous genes confer resistance to various drugs or inhibitors instead of leading to respiratory deficiency. Those include resistance to antimycin A, mucidin, diuron or funiculoson for the *cob* gene or resistance to oligomycin for the *oli1* and *oli2* genes.

Respiration deficient mutants may also result from point mutations in the genes of the protein synthetic machinery (tRNA or rRNA genes). Such mutants (syn^-) lead to partial or conditional deficiency in all mitochondrial gene products (complete deficiency causes a high frequency of rho^- formation. The above described mutants have been used to construct a genetic map of the mitochondrial genome of *S. cerivisiae* which has been correlated with restriction maps and sequence data of mtDNA from various strains. Since there is no complete DNA sequence from a single strain the present map is a patchwork of data from different strains (see figure 2).

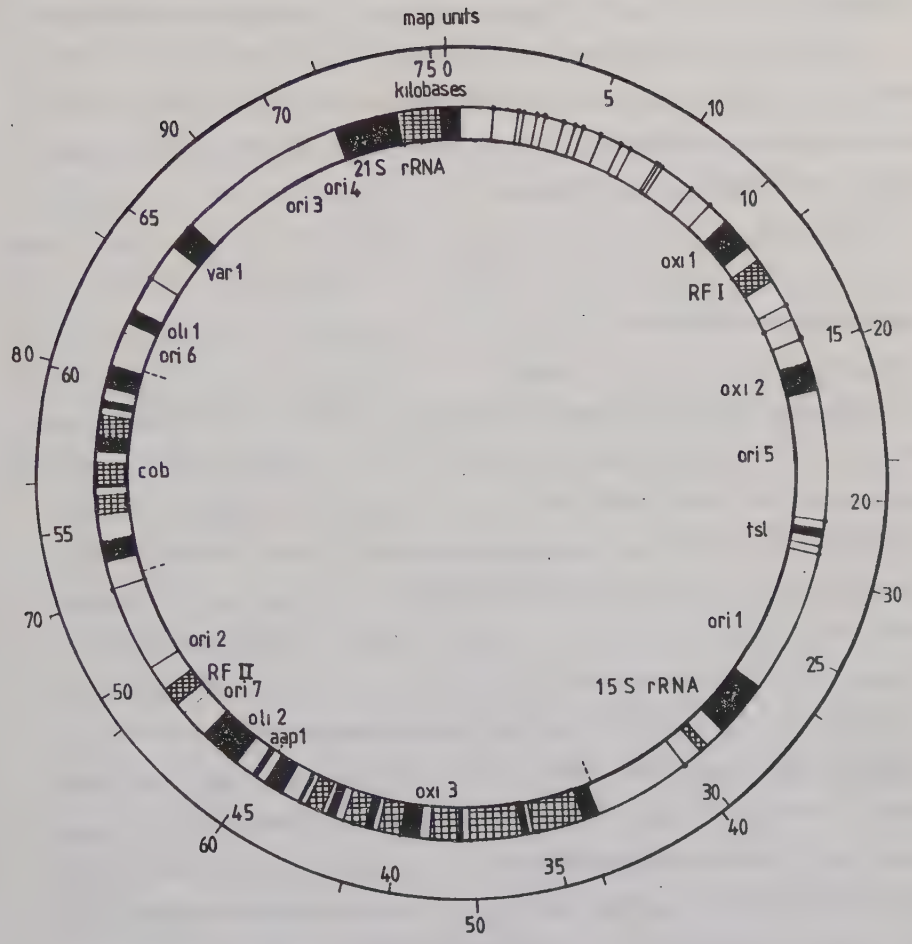

Figure 2. Map of the mitochondrial genome of the yeast Saccharomyces cerevisiae . The map has been adapted and updated from Dujon, 1983. Exon sequences are denoted by black segments. The hatched segments represent open reading frames with imprecisely defined functions. tRNA genes are denoted by thin radial lines. The direction of transcription is clockwise for all genes except $tRNA^{thr1}$. tsl is the tRNA synthesis locus (see discussion). The positions of the seven putative origins of replication (ori sequences) are only approximate and have been obtained by comparing the map of Goursot et al, 1983, with that of Dujon et al., 1983).

Natural polymorphism also offers a means to investigate the mitochondrial genome of S. cerivisiae . Polymorphic variations are frequent between various strains. They mainly concern the presence or absence of introns and of G+C rich clusters, but protein genes can also exhibit intriguing size variations (e.g. var1 , Hudspeth et al., 1982).

Objectives and strategy

The aim of this work was to obtain a greater understanding of some of the processes involved in the transcription and processing of the gene for apocytochrome b (cob) in yeast mitochondria. Previous studies performed in this institute have concentrated on the processes involved in the removal of introns from the cob pre-mRNA. At the outset of this dissertation little was known about the processes involved in the production of some of the earlier intron containing pre-mRNA species. In addition the size of the cob transcription unit was also poorly defined. Thus, it was uncertain as to whether the cob gene was transcribed separately as a mono-, or with some of the surrounding mitochondrial genes, as a poly-cistronic transcript. The initial objective of this dissertation was to characterise some of the early post-transcriptional events of the cob region with a view to defining the sequence content of the cob primary transcript. Subsequent analysis of various mutants of this region would hopefully then provide a clue as to the nature of the sequences involved in correct processing of the cob transcripts. Previous studies on intron processing have made use of a large collection of mit^- strains. Screening of this

collection revealed only one strain (M9410) disturbed in intergenic processing events. An alternative strategy was therefore utilised which relied on the rho^- phenomenon (described above) of yeast mitochondria. The use of such strains allowed two problems to be tackled simultaneously: firstly, by comparison of rho^- and wild type transcription the degree of dependence of mitochondrial transcription and RNA processing on mitochondrial translation was assessed. Secondly, the analysis of accumulated processing intermediates revealed which processing steps required mitochondrially encoded factors (protein or RNA) not encompassed by the mitochondrial genome of the rho^-.

II. Materials and methods

1. Genetic symbols

rho^+

wild type mitochondrial genome

rho^-

respiratory deficient mitochondrial deletion mutant containing an excised and amplified segment of the parental genome.

rho^o

respiratory deficient mutant lacking the complete mitochondrial genome

mit^-

point mutation or small deletion of mitochondrial DNA

wt

wild type

2. Strains

Strain	Genotype Nuclear	Mitochondrial	Origin
777-3A	alpha, ade2 , op1	rho^+ mit^+	Kovacova et al. (1968)
D 273-10 B	alpha	rho^+ mit^+	Sherman et al. (1978)
KL 14-4A	a, his1 , trp2	rho^+ mit^+ cap^r-321 oli^r1-1 par^r-454	Wolf et al. (1973)
dD22 BC5	a, ura3 , trp, tsp25	rho^- from RM511-4A	
M1301 M5020 M9410 M561 M9391 M4457	alpha, ade2 , op1	rho^+ mit^- from 777-3A	Schweyen et al. (1978)

3. Chemicals

Acrylamide	Serva, Heidelberg
Agar	Sigma, St. Louis USA
Bisbenzimide	Riedel de Haen, Hannover
Bovine serum albumin	Sigma, St. Louis USA
Bromophenol blue indicator	E. Merck, Darmstadt
Caesium chloride	Baker chemicals
DMSO	EGA
DNA from herring sperm	Sigma
EDTA	Baker chemicals
Hydrazinium hydroxide	Merck-Schuchardt,Munich
Ficoll 400	Serva
Formamide	Sigma
Glyoxal	EGA-Chemie, Steinheim/Albuch
N.N. Methylenebisacrylamide	Serva
Polyvinylpyrrolidone	Serva
Sodium lauryl sulphate	Serva
Sodium citrate	Serva
TEMED	Serva
Tris	Sigma
Zymolase	Kirin Brewery Ltd., Japan

4. Radio-isotopes

^{32}P dNTP, ^{32}P NTP	Amersham-Buchler, Braunschweig.
	New England Nuclear, Dreieich, BRD

5. Enzymes:

Nucleic acid labelling :

DNA polymerase I	Boehringer Mannheim
DNA polymerase, Klenow fragment	Boehringer Mannheim
Polynucleotide Kinase	Boehringer Mannheim
S1 Nuclease	Boehringer Mannheim
Guanylyltransferase	Bethesda Research Laboratories, USA

Restriction endonucleases :	Boehringer Mannheim
	P-L Biochemicals, St. Goar BRD Biolabs
	BRL, Maryland, USA

Other :

Proteinase K	E. Merck

6. Abbreviations

A	adenosine
AMP	adenosine 5'-monophosphate
AP	ammonium persulphate
ATP	adenosine 5'-triphosphate
bp	base pair
BSA	bovine serum albumin
cpm	counts per minute
C	cytidine
cytDNA	cytoplasmic DNA
DMSO	dimethylsulphoxide
dATP	deoxyadenosine 5'-triphosphate
DNA	deoxyribonucleic acid
EB	ethidium bromide
EDTA	ethylenediaminetetra-acetate
G	guanosine
GTP	guanosine 5'-triphosphate
mRNA	messenger RNA
mitRNA	mitochondrial RNA
Pipes	1,4-piperazinediethanesulphonic acid.
p	phosphate group
RNA	ribonucleic acid
RNase	ribonuclease
rRNA	ribosomal RNA
r.p.m.	revolutions per minute
S	Svedberg unit, the sedimentation coefficient
SDS	sodium lauryl sulphate
T	thymidine
TE	Tris-EDTA

TEB	Tris-EDTA-borate
TEMED	N,N,N',N-tetramethyl ethylenediamine
Tris	tris(hydroxymethyl)aminoethane
tRNA	transfer RNA
U	uridine
uv	ultra violet radiation

7. Media and buffers

Solid media:

YD

In 1l desalinated water :

Yeast extract	1%	10g
Agar	2%	20g
Glucose	3%*	

(*added as a sterile 50% solution after autoclaving.)

YGal

As for YD except that galactose is added to 3% end concentration as a sterile 30% solution after autoclaving.

YG

As for YD except 3% glycerol is added prior to autoclaving.

Liquid media

YD,YG and YGal

As for solid media except for the exclusion of agar.

YE 10%

In 1l desalinated water:

Yeast extract	100g

Autoclaved for 30 mins.

1M Phosphate buffer

NaH_2PO_4	1M
Na_2PO_4	1M

Mixed according to required pH

TEB buffer x 10 (pH 8.3)

Tris	0.9M
Boric acid	0.9M
EDTA	2.5mM

20 x SSC

Sodium chloride	3M
Sodium citrate	0.3M

8.Methods

8.1. Isolation of mt DNA

Mitochondrial DNA was isolated as described by Lang et al., (1977) except that the crude mitochondrial pellet was lysed in a buffer containing 50 mM Tris-HCl pH 7.5 , 2 mM EDTA, 2% SDS and 10 ug/ml proteinase K. The lysate was incubated at 30°C for 1 hour to allow digestion of the contaminating proteins by the proteinase K. Protein was precipitated by adjusting the lysate to 1 M NaCl and allowing it to stand for at least 2 hours at 4°C followed by centrifugation at 15 000 r.p.m. for 20 mins. Removal of contaminating nuclear DNA was carried out by CsCl density gradient centrifugation as described by Grosch (Ph.D thesis, 1980), except that removal of the CsCl was carried out by the addition of an equal volume of T.E. followed by the addition of three volumes of ethanol. The solution was allowed to stand at room temperature for one hour during which time precipitation of the DNA occurred. The precipitate was pelleted by centrifugation at 15 000 r.p.m. for 10 minutes. The pellet was redissolved in 50 mM Tris pH 7.5 and reprecipitated with a solution of 90% ethanol, 0.3M ammonium acetate.

8.2. Restriction and gel-electrophoresis of mtDNA

DNA was digested with restriction enzymes as described by the manufacturer. Gel electrophoresis was carried out as described by Grosch (1980).

8.3. Isolation of mtDNA restriction fragments

DNA fragments were recovered from agarose and acrylamide gels by electro-elution.

8.4. Radioactive labelling of mtDNA

8.4.1. Nick Translation of mt DNA

Labelling was carried out according to Rigby et al.(1977) except that the DNase treatment was omitted. The nonincorporated triphosphates were removed by passing the reaction mix through a sepharose G-50 column (4 ml).

8.4.2. 5' end labelling with Polynucleotide kinase

Prior to labelling, terminal 5' phosphates were removed by treatment with calf intestinal phosphatase (CIP) (Maniatis et al., 1982). Polynucleotide kinase and (^{32}P)ATP were used to end label DNA by forward phosphorylation of protruding 5'-hydroxyls (Maniatis et al., 1982).

8.4.3. 3' end labelling with DNA Polymerase

The Klenow fragment of E. coli DNA polymerase was used to label recessed 3' ends of double stranded DNA (Maniatis et al., 1982)

8.5. Southern analysis of mtDNA

Southern analysis was carried out according to Southern (1975).

8.6. DNA sequence analysis

End labelled DNA fragments were subjected to chemical degradation as described by Maxam and Gilbert (1980). The reaction products were separated on a 50 cm long, 0.5 mm thick gel with an acrylamide concentration of 20%, 8%, or 6%. The gels were exposed to Kodak XR 5 X-ray film at -70°C.

8.7 Strand separation of mt DNA

Strand separation by denaturation and gel electrophoresis was carried out according to Maxam and Gilbert (1980).

8.9. Isolation of mt RNA

Mitochondria were isolated from protoplasts and RNA was extracted from purified mitochondria as described by Morimoto et al., (1979) . Mitochondrial DNA was removed either by centrifugation in CsCl or precipitation in LiCl:

(i) Caesium chloride (CsCl) gradient method

The RNA, dissolved in 1 ml of TE, was loaded onto 2.7 ml of CsCl and centrifuged overnight at 40 000 r.p.m. in an SW 60 Ti. The addition of ethidium bromide facilitated the visualisation of

the RNA pellet. The pellet was dissolved in 0.5 ml of TE and precipitated with ethanol and 0.3 M sodium acetate.

(ii) Lithium chloride (LiCl) method

The RNA was dissolved in 1 ml distilled water and adjusted to 2 M LiCl by the addition of an equal volume of 4 M LiCl. The solution was allowed to stand overnight at 4°C. The RNA was recovered by centrifugation at 10 000 r.p.m for 10 minutes. The supernatant which contained DNA and tRNA was discarded.

8.10. Northern analysis of mtRNA

Northern analysis of glyoxalated mtRNA was carried out according to Thomas (1981). S values of transcripts and their corresponding sizes in bases were determined by calibration with 15S mitochondrial rRNA (1,600 nucs.) and 21S mitochondrial rRNA (3200 nucs.) (Tabak et al., 1979).

8.11. <u>In vitro</u> capping of mt RNA

RNA was incubated for 30 minutes at 37°C in the following buffer:

50 mM Tris-HCl, pH 7.9
1.25 mM $MgCl_2$
2.5 mM DTT
50 pm (alpha-^{32}P) GTP 3000 Ci/mmol
0.1 U Guanylyltransferase.

Non-incorporated triphosphates were removed by gel-filtration through a 2 ml column of bio-gel P40 equilibriated with 100mM NaCl 50 mM Tris-HCl, pH 7.5, 2 mM EDTA and 2 % SDS. Peak fractions were collected and concentrated by precipitation in 90 % ethanol-0.3M ammonium acetate.

III. Results

1. Detailed physical characterisation of the $tRNA_{glu}$- cob segment of yeast mtDNA

1.1. Utilisation of a rho^- clone for the isolation of sequence specific probes

Prior to investigating transcription of the $tRNA_{glu}$- cob region of the yeast mitochondrial genome a detailed restriction endonuclease map was constructed. Construction of such a map was particularly necessary for the isolation of specific DNA probes for transcript analysis. The region to be investigated spanned some 5kb which represents about 6% of the total genome. For transcript analysis probes of about 0.5kb in size were to be isolated which would represent some 0.6% of the total genome. It was therefore considered impractical to isolate DNA probes from the wild type genome. Instead, use was made of the rho^- petite strain BC5. Rho^- petites are respiratory deficient macro-deletion mutants in which an excised segment of the wild type genome has been amplified (tandemly or palindromically) so that the excised segment becomes the repeat unit of the rho^- strain (fig. 1). Genetic data showed that the rho^- strain

BC5 possessed a segment of the mt genome mapping from cob intron 1 to a position between cob and oli2 (fig. 3). Preliminary restriction digest data showed the repeat unit of BC5 to be about 6kb, extending about 4kb upstream from the cob region. This presents one with an inbuilt cloning system allowing the isolation of large quantities of mtDNA from selected regions of the genome.

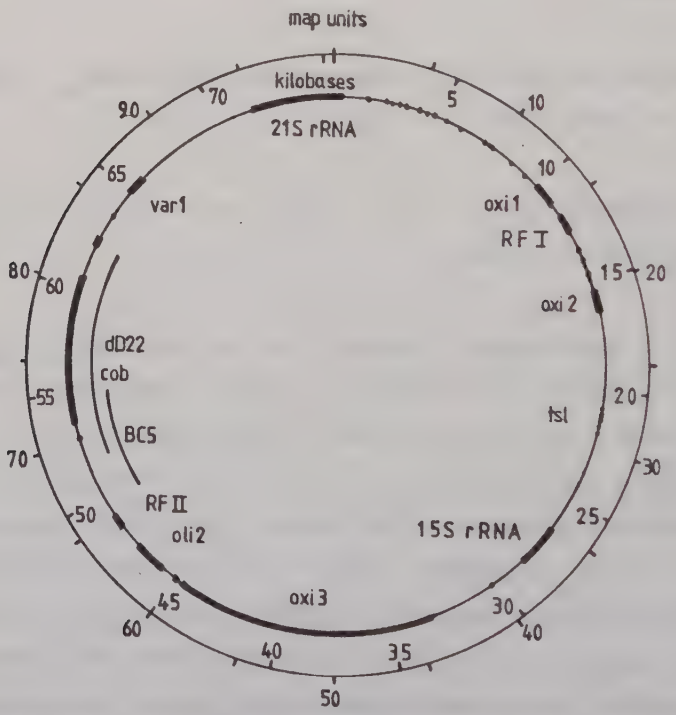

Fig. 3: Schematic representation of the region encompassed by the rho⁻ genome of BC5. The repeat unit of BC5 exists as a tandemly amplified mtDNA segment of about 6kb and encompasses a region extending from cob intron 1, upstream to a site 5' of ori2 (see genome map, fig. 2).

1.2. Detailed restriction site mapping of the $tRNA_{glu}$- *cob* genome segment

1.2.1. Mapping strategy

Two independent yet complementary approaches were taken to construct the restriction map of BC5. The first approach entailed two step digestion of BC5 DNA with two different enzymes. Thus purified mtDNA from BC5 was first digested with either Hinf I or Mbo I. The resulting fragments were separated on an agarose gel, the individual fragments excised from the gel and eluted. Each fragment was then separately redigested with one of the 11 restriction enzymes listed below. The digest was rerun on a second agarose gel. Analysis of the two sets of restriction digest data allowed the ordering of the HinfI, MboI, AluI, Rsa and HindII restriction enzyme sites. This data was both confirmed and extended with the data obtained by the second approach. This entailed the binding of electrophoretically separated restriction digests onto nitro- cellulose filters. The bound DNA was probed with radioactive ^{32}P DNA restriction fragments isolated from BC5. Using this method restriction digest fragments lying within the sites defining the ^{32}P DNA probe used could be unambiguously identified.

1.2.2. Double digest restriction mapping of BC5

1.2.2.1. Single digests of BC5 mitDNA

Mitochondrial DNA from the rho⁻ strain BC5 was digested with the restriction enzymes listed in table 3 and run on a 1.5% agarose gel using phiX 174 digested with Hae III as a marker. The estimated length in base pairs of each fragment is shown in table 4.

Restriction enzyme	Recognition site
Alu I	5'-AGCT-3' 3'-TCGA-5'
Aha III	5'-TTTAAA-3' 3'-AAATTT-5'
Bgl I	5'-GCCNNNNNGGC-3' 3'-CGGNNNNNCCG-5'
Hae III	5'-GGCC-3' 3'-CCGG-5'
Hind II	5'-GTPyPuAC-3' 3'-CAPuPyTG-5'
Hinf I	5'-GANTC-3' 3'-CTNAG-5'
Hpa II	5'-CCGG-3' 3'-GGCC-5'
Hph I	5'-GGTGANNNNNNNN-3' 3'-CCACTNNNNNNN -5'
Mbo I	5'-GATC-3' 3'-CTAG-5'
Rsa I	5'-GTAC-3' 3'-CATG-5'
Xba I	5'-TCTAGA-3' 3'-AGATCT-5'

Table 3: Restriction endonucleases used to construct a restriction site map of BC5 rho⁻ mtDNA.

Alu I	Aha III	Hha I	Hae III	Hind II	Hinf I
4250	1825	4400	3225	5600	1700
1150	1100	1200	900		1150
200	600		850		950
	550		400		900
	500		225		500
	475				400
	450				
	100				

Hpa II	Hph I	Mbo I	Rsa I	Xba I	Bgl I
1900	3150	2250	3650	uncut	uncut
900	1200	1750	1500		
775	600	900	450		
700	250	700			
400					
375					
275					
200					
100					

Table 4: Length in base pairs of single restriction enzyme digests of mtDNA of the rho$^-$ BC5.

1.2.2.2. Redigestion of Hinf I and Mbo I fragments

To determine the relative position of the fragments observed in the above digests, the individual Hinf I and Mbo I fragments were isolated and redigested with all of the enzymes used in the single digests. Tables 5 and 6 give the sizes of the restriction fragments obtained by recutting the Hinf I and Mbo I fragments.

Hinf I fragment:	1700	1150	(950 900)	500	400
Recut with					
Alu I	1700	1150	900 600 350	200 175 125	400
Aha III	1100 500 100?	550 450 150	600 450 350 300 100 50?	500	400
Bgl I	1700	1150	950 900	500	400
Hae III	900 600 200	775 375	950 550 350	500	400
Hha I	1700	775 375	950 900	500	225 175
Hind II	1700	700 450	950 900	500	400
Hpa II	775 350 275 200 100 50	400 375 375	625 550 375 325	500	400
Hph I	1700	1150	900 600 350	250 250	400
Mbo I	900 650 125?	1150	600 300 950	250 250	400
Rsa I	1350 350	1150	950 900	500	400
Xba I	1700	1150	950	500	400

Table 5: The estimated length in base pairs of the restriction fragments obtained by recutting the Hinf I fragments with the enzymes listed in table 3.'?' indicates that the fragment could not be clearly detected.

Mbo I Fragments : Recut with	2250	1750	900	700
Alu I	1150 1025 75	1650 100	900	700
Aha III	950 600 550 150	825 475 450	900	550 100? 50?
Bgl I	2250	1750	900	700
Hae III	2150 100	1050 700	800 100 100	400 200 100
Hind II	2250	1050 675	900	700
Hinf I	950 650 400 250	1175 300 250	900	575 125
Hpa II	smear	650 575 400	700 100 100	400 100 50?
Hph I	1250 600 400	1200 250 300	900	700
Rsa I	1500 450 300	1750	900	700
Xba I	2250	1750	900	700

Table 6: Estimated length in base pairs of the restriction fragments obtained by recutting the Mbo I fragments of BC5 mtDNA.

Figure 4 shows an example of secondary digests of the BC5 Hinf I and Mbo I fragments using the restriction enzyme Aha III.

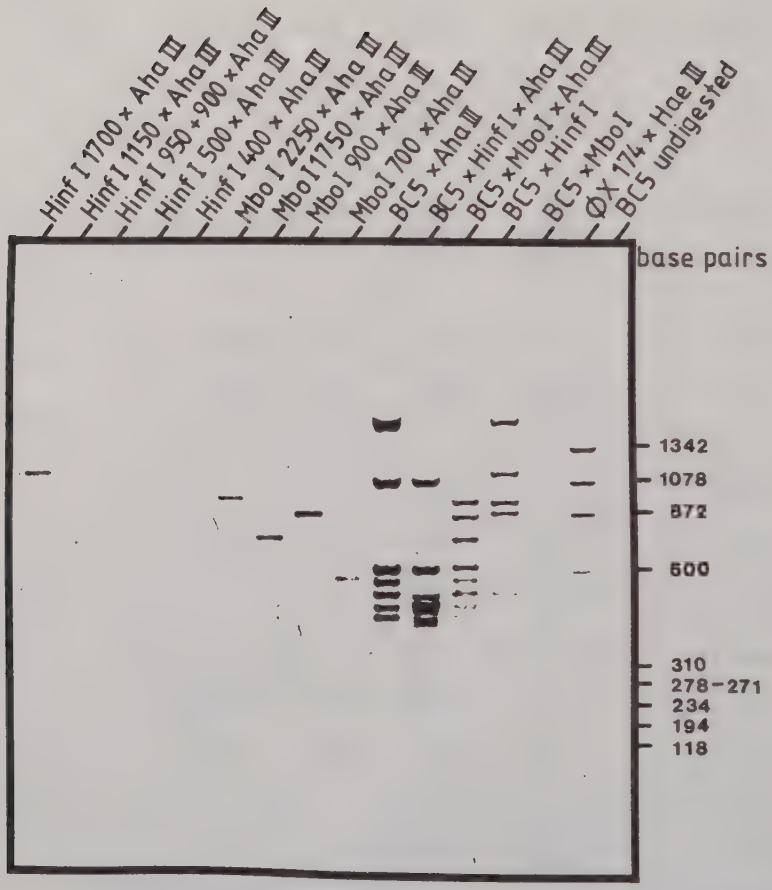

Figure 4: Example of a secondary digest analysis used in the construction of a restriction site map of the rho⁻ genome BC5. Hinf I and Mbo I digest fragments were recut with Aha III and run on a 1.5% agarose-TEB gel.

The above data allowed the construction of a restriction enzyme site map for Hinf I, Mbo I, Alu I, Hind II and Rsa I. Figure 5 shows how the single and secondary digest data for Alu I, Hinf I

and Mbo I were combined to determine the sequence of the fragments and sites within the BC5 genome. It should be noted that the enzymes Bgl I and Xba I failed to cut in both the single and secondary digest experiments which indicated the absence of the corresponding restriction enzyme recognition sites.

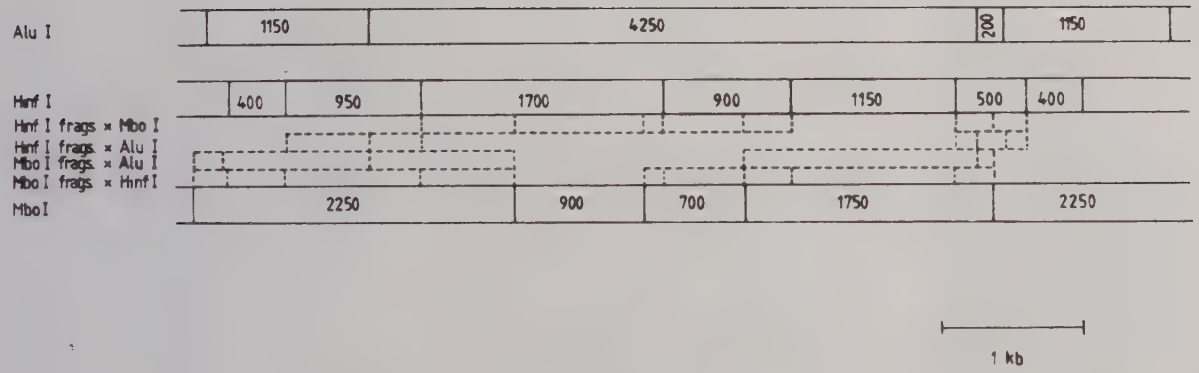

Figure 5. Construction of a restriction enzyme site map of BC5 using the enzymes Alu I, Mbo I and Hinf I. The single digest fragments were placed in sequence such that adjacent sites would produce DNA fragments of the size seen in the secondary digest experiments.

1.3. Restriction mapping using the Southern blot hybridisation method

1.3.1 Preparation of filter bound restriction digests of rho$^-$ BC5 DNA

Single digests of mtDNA from BC5 were electrophoresed on 1.5% agarose-TEB gels and transfered to nitrocellulose paper. Ten different single digests were run in parallel on one gel. Seven such gels were run.

1.3.2. Preparation of ^{32}P DNA probes

To ease interpretation of the data it was important that the restriction fragments used to probe the filter bound DNA were not contaminated with other fragments. Probes were therefore isolated by using a two step procedure: mitochondrial DNA from BC5 was digested with Mbo I. The resulting fragments were eluted and recut with a second enzyme (Hinf I or Hae III). This digest was rerun on a second gel. The required fragments were eluted, precipitated in ethanol and labelled by nick translation. The following table summarises the isolation procedure for each of the seven probes used.

Probe	Mbo I fragment	second enzyme and fragment	
A	2250	Hinf I	400
B	2250	Hinf I	950
C	2250	Hinf I	600
D	900	Hae III	800
E	700	Hinf I	575
F	1750	Hinf I	1150
G	2250	Hinf I	250

Table 7: ^{32}P DNA probes used in restriction mapping of BC5 mtDNA. The above ^{32}P-DNA were hybridised to the filter bound restriction digests.

1.3.3 Analysis of hybridisation data

The following table summarises the hybridisation data. The table shows which fragment of each restriction digest hybridised against each ^{32}-P probe.

	^{32}P DNA probes						
BC5 digest	A	B	C	D	E	F	G
Aha III	1825	1825 600 55	1150 550	1100	500 100	1825 475 450	1825
Alu I	1150	4250	4250	4250	4250	4250	1150 200
Hae III	3225	3225	3225 850	850	900 400 225	3225 900	3225
Hinf I	400	950	1700	1700	900	1150	500
Hpa II	1900	---	700 275	775	400	1900 900 400	1900
Hph I	600 400	3150 400	3150	3150	3150	1200	600
Mbo I	2250	2250	2250	900	700	1750	2250
Rsa I	450	1500	1500	3650	---	3650	450

Table 8: Summary of Southern hybridisation data. BC5 mtDNA was digested with the enzymes listed and hybridised against the ^{32}P BC5 mtDNA probes (A-G) listed in table 7.

Figure 6 shows an example of an autoradiogram of a ^{32}P labelled probe hybridised to filter bound restriction digests of BC5.

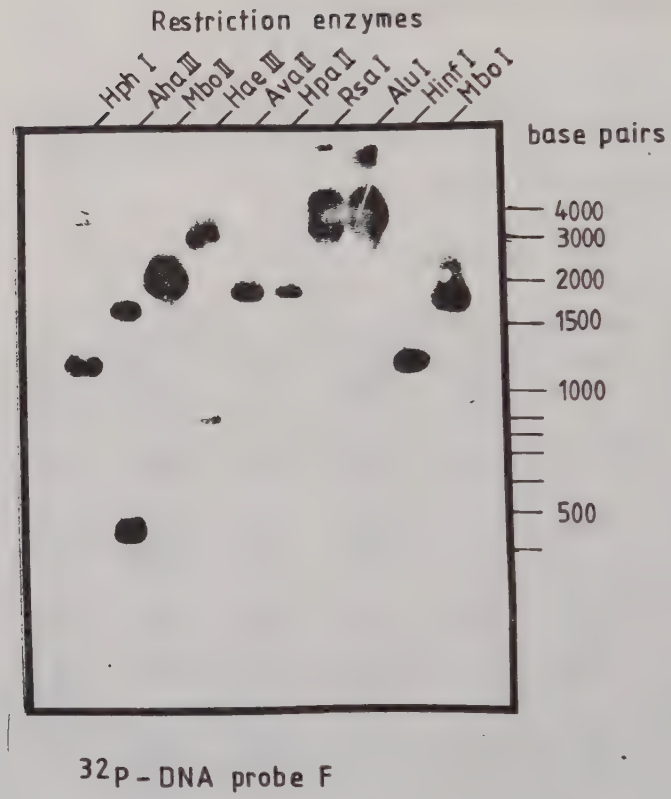

Figure 6. Restriction site mapping using the Southern blot hybridisation method. Example of an autoradiogram showing the hybridisation pattern of a nick-translated 1150 bp HinfI restriction fragment, isolated from the rho⁻ strain BC5, and hybridised against filter bound restriction enzyme digests of BC5.

The above data allowed the construction of a restriction site map as shown in figure 7. Of interest is the frequency of Hpa II and Hae III site and the way in which they coincide. Sequence analysis has found this to be due to the occurrence of GC rich clusters in the yeast mitochondrial genome which

characteristically contain Hpa II - Hae III site clusters (Tzagoloff et al. 1980).

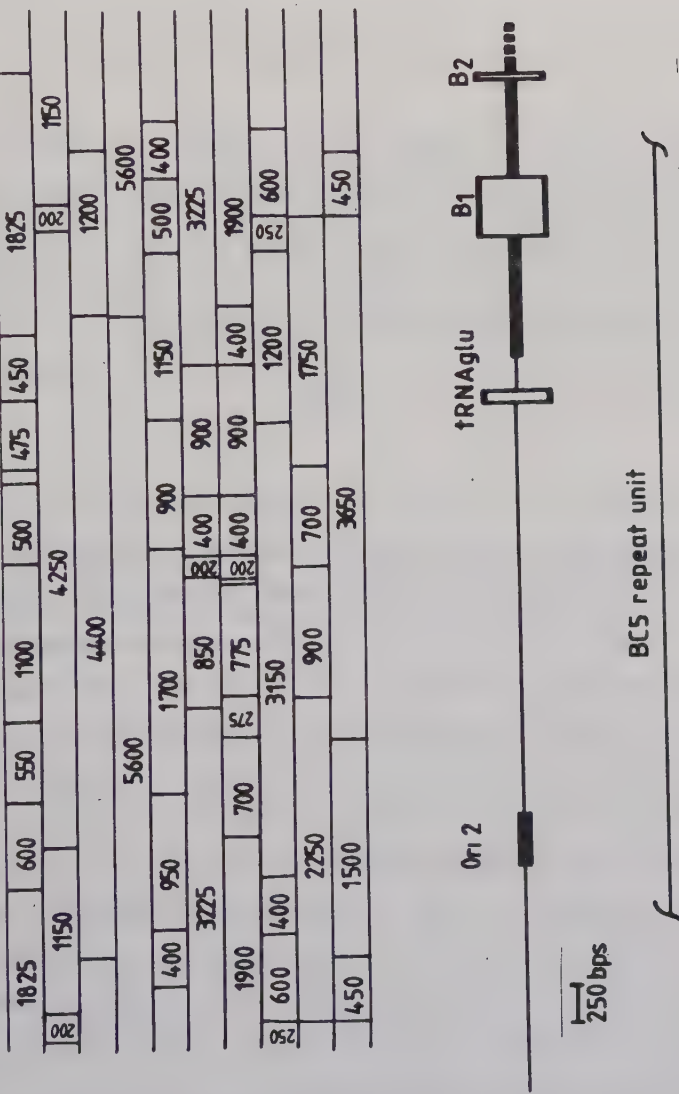

Figure 7. Restriction map of [illegible] unit of the rho⁻ strain BC5. The map was constructed by analysys of single (table 4) and secondary digests (tables 5 and 6) of BC5 mtDNA combined with ^{32}P data obtained by Southern analysis of restriction enzyme digests using ^{32}P BC5 mtDNA restriction enzyme fragments (table 7).The junction region of the repeat unit of BC5 is defined by the HinfI 400 bp fragment. The restriction fragments from the neighbouring repeat units are also shown. The restriction enzyme map was aligned with the coding sequences of the cob gene as determined by DNA sequence analysis (Bonitz et al., 1982).

1.4. Comparison of restriction map with published data

The restriction map obtained for BC5 was compared with the restriction map of Goursot et al., (1982). Good agreement was found with most sites, differences being in the order of 50 bp which is an acceptable discrepancy considering that the resolution of the gel system used was in the range of 20 - 50 bp and considering that the two maps were produced in different labs.

Large differences were seen with the enzymes Hpa II and Hae III. Thus in places sites were either missing or appeared to be displaced by more than 50 bp and some double sites were only present as single sites. There are several possible explanations for these observations. Firstly, double sites may have only been detected as single sites due to the intervening distance being less than 50 bp i.e. lower than the resolution of the gel system used. Secondly, the main differences were seen with Hpa II and Hae III which are characteristic of the GC rich clusters found in yeast mitochondria. Other authors (Sor and Fukuhara, 1982) have noted that the presence or absence of such sequences are typical interstrain differences . Thus, the differences between the two sets of data could well be due to the difference in distribution of such GC rich sequences.

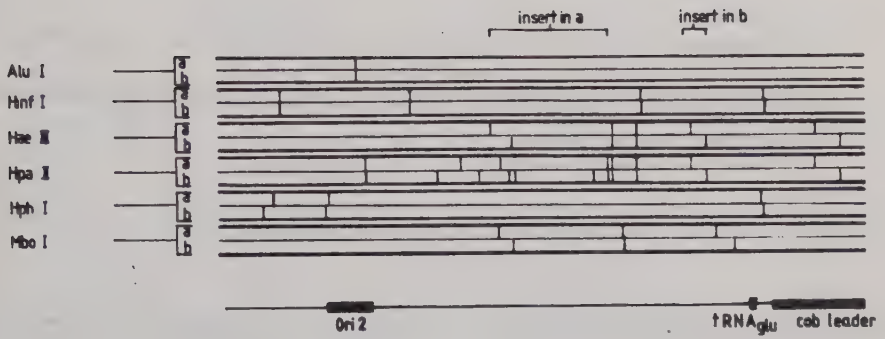

Fig. 8 Comparison of the restriction site map of the rho$^-$ strain BC5 (a) and the rho$^-$ strain bi7 (Goursot et al., 1982) (b). The two sets of data were aligned so as to detect any major differences between the two strains.

2. Detection and analysis of a DNA polymorphism between $tRNA_{glu}$ and its promoter

2.1. Insert polymorphisms in yeast mitochondria

The S. cerevisiae mitochondrial genome exhibits several forms of sequence polymorphisms. One such polymorphism involves the strain dependent possession of intervening sequences (introns). For example cob introns one, two and three of the gene for apocytochrome b in strain 777-3A are absent in strain D273 (fig 9). The second form of insert is much shorter (20 - 40 bps) and is characterised by the presence of GC rich sequences bearing HpaII and HaeIII clusters. Such a sequence is sometimes found in the central part of the small ribosomal RNA (15S RNA) of yeast mitochondria (Sor and Fukuhara, 1982).

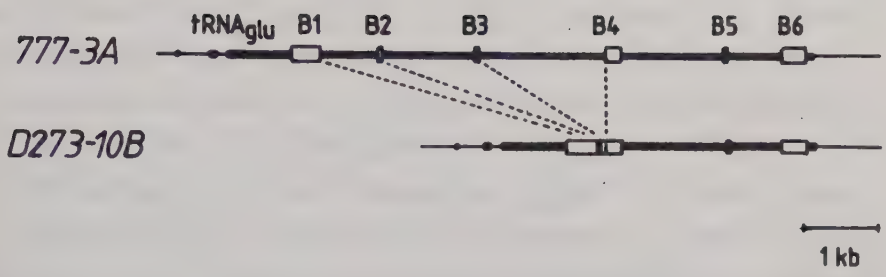

Fig. 9. Schematic comparison of long and short cob genes in yeast mitochondria.

2.2. Detection of a mini-insert adjacent to the $tRNA_{glu}$ gene

This dissertation has focussed on the $tRNA_{glu}$ - cob segment of the yeast mitochondrial genome. Bonitz et al. (1982) published the sequence encompassing the tRNA and the leader of cob in the short strain D273-10B. However, the strains used in this thesis were all long form and therefore not necessarily identical in sequence content to the published sequence. The sequences around the tRNA in short and long form strains were therefore compared by restriction analysis. 777-3A and D273-10B mtDNA Hinf - Mbo I double digests were transferred to nitrocellulose and probed with a 300 bp Hinf - Mbo I DNA fragment, isolated from rho^- BC5, containing most of the glutamic tRNA gene. Figure 10 shows the resultant

autoradiography, from which it can be seen that the ^{32}P probe hybridised against a 300 (+/- 25 bp, limit of resolution of gel system used) fragment in the long form strains (777-3A, BC5 and dD22) but to a 235 bp fragment (according to sequence data of Bonitz et al., 1982) in the short form strain (D273-10B). This difference is interpretated as indicating the presence in the long form strains of an additional 65 (+/- 25 bp). The ^{32}P probe used was defined by a Hinf site, -15 from the 3' end of $tRNA_{glu}$, and an Mbo I site, -325 from the 3' end of $tRNA_{glu}$. Thus, the insert seen in the long form relative to the short form could be located either upstream from or within the $tRNA_{glu}$.

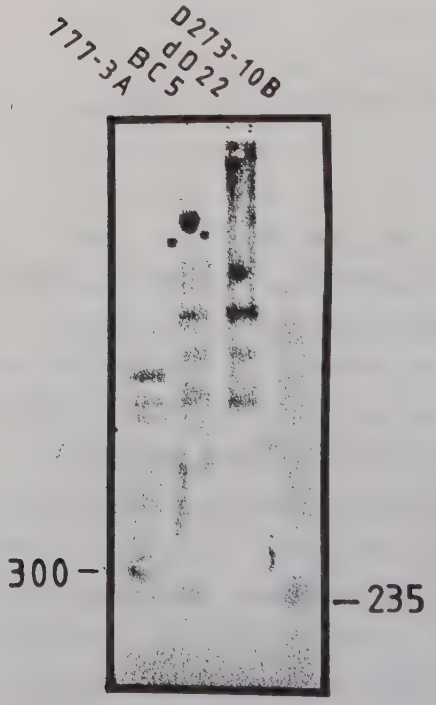

Fig. 10. Location of an insert between the $tRNA_{glu}$ and its promoter. Nitrocellulose bound Hinf-Mbo I double digests were probed with a 300 bp Hinf-Mbo I fragment isolated from BC5. It can be seen that the 300 bp fragment in BC5, dD22 and 777-3A corresponds to a 235 bp fragment in D273-10B.

2.3. Sequence analysis of the insert

On the basis of the above data it was not possible to say whether the insertion lay within or outside the gene for $tRNA_{glu}$. The precise location of this insert was of upmost interest, since there is to date no known example of a split mitochondrial tRNA gene. The sequence content of the insert was also of interest since other common small DNA sequence inter-strain polymorphisms have been found to be due to the presence of GC rich sequences characteristically bearing HpaII-HaeIII clusters. The insert reported here lacked such sites and so probably represented a different class of insert. A HpaII-HinfI 450 bp fragment, bearing the above characterised insert was isolated from BC5 mtDNA, end labelled with ^{32}P-alpha-dATP using Klenow polymerase and sequenced according to Maxam and Gilbert (1980). The sequence data allowed a precise localisation of the insert to a position 147 bps upstream from the glutamic tRNA. The insert was seen to consist of runs of 3-4 Ts interupted by As, which is almost identical to the flanking 3' sequence (fig. 11). Thymidine rich sequences represent 'hot spots' for deletion events (Fox and Weiss-Brummer, 1980 and Novitski et al., 1984, Weiss-Brummer pers. com.). It appears that such sequences are particularly prone to 'slipping', either of the DNA polymerase along the DNA or of one DNA strand against the other (see discussion). It should be stressed that the term insert is used loosely to denote a DNA sequence polymorphism and not to indicate that the polymorphism arose by an insertion event; it could equally well have arisen by a deletion event.

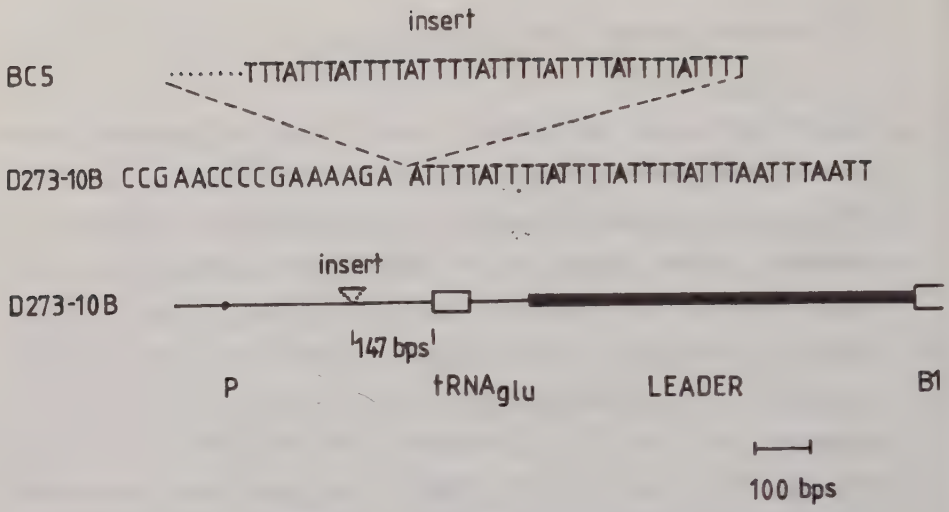

Figure 11: Diagramatic presentation of a DNA sequence polymorphism showing its precise position in relation to the glutamic tRNA gene and its DNA sequence and that of the flanking region common to both strains.

2.4. Position of the $tRNA_{glu}$ promoter

Christianson et al., (1983) have reported that the promoter for the $tRNA_{glu}$ gene lay 391 bps upstream. This figure was arrived at by aligning the sequence of an <u>in vitro</u> capped oligonucleotide from the strain MH41-7B (long form) with the published sequence data (Bonitz et al. 1982) from the strain D273-10B (short form). The above data show that the long form strains bear an insert of about 65 (+/-25) bps, 147 bps upstream from the $tRNA_{glu}$ and that accordingly, in the long form the promoter is situated at - 456 +/-25 bps and not -391 as reported by Christianson et al., (1983).

2.5. The 9S $tRNA_{glu}$ precursor bears an original 5' terminus

In section 3.7.3 a 9S transcript containing $tRNA_{glu}$ sequences was characterised. Comparison of the mobility of this transcript with the mobility of the 4S $tRNA_{glu}$ (75 nucleotides, Bonitz et al., 1982) and the 15S rRNA (1600 nucleotides, Tabak et al., 1979) indicates that this transcript is about 500 nucleotides in length. It is considered to exist as a $tRNA_{glu}$ transcript bearing a 5' extension relative to the mature $tRNA_{glu}$. It was demonstrated above that the $tRNA_{glu}$ promoter probably lies about 465 bps upstream. The $tRNA_{glu}$ itself is 75 bps long. It, therefore, seems likely that the 9S transcript observed in the rho^- strain dD22 represents a $tRNA_{glu}$ precursor bearing a 5' terminus corresponding to the site of transcriptional initiation.

3. Inter and intragenic processing events of the $tRNA_{glu}$-*cob* region

3.1. Genome transcription

Although only a fraction of the yeast mitochondrial genome codes for known products (Borst and Grivell, 1978), almost a full single strand equivalent is transcribed into RNA. Some of the genes coded by mitochondrial DNA contain one or more optional introns. This contributes to the complex array of transcripts generated during extensive splicing and processing steps (Schmelzer et al., 1981, Van Ommen, 1980, Levens et al. 1981). The aim of this study was to extend our knowledge of yeast mitochondrial transcription and RNA processing by a detailed analysis of the transcripts of the $tRNA_{glu}$-*cob* genome segment of yeast mtDNA. In light of the knowledge that mitochondria encode only a fraction of the components required for mitochondrial biogenesis, it was of interest to determine to what extent mtRNA transcription and processing relied on mitochondrially encoded factors :

i) Intragenic processing events. This covers intron splicing, the aim being to characterise those processes that required no mitochondrially encoded factors.

ii)Intergenic processing events. This includes both trimming events and those processes involved in the processing of cotranscripts to their constituent gene precursors. An ancillary

aim was also to determine which processes required the participation of mitochondrially encoded factors.

3.2.1. Use of a rho$^-$ strain to investigate cob transcription

The approach chosen made use of the rho$^-$ petite phenomenon of yeast. Rho$^-$ petites are macro-deletions of the mitochondrial genome, whereby the retained segment is amplified several times to form the circular rho$^-$ genome (fig. 1). Rho$^-$ mitochondria characteristically lack a functional translation system and are therefore incapable of synthesising mitochondrially encoded proteins. Any RNA processing carried out in rho$^-$ mitochondria must be carried out either by proteins imported from the cytoplasm or by the RNA molecules themselves (Kruger et al., 1982 and Guerrier-Takada et al., 1983). To examine the contribution made by cytoplasmic factors transcripts produced by wild type strains and a cob rho$^-$ (dD22) were compared.

3.2.2. Physical mapping of dD22

The rho$^-$ strain dD22 had been previously characterised by Grosch et al., (1981). It had been shown to span the complete cob region. Subsequent restriction analysis also demonstrated that it contained the gene for the glutamate tRNA (fig. 13).

3.2.3. Comparison of wild type and the rho$^-$ strain genome

Before initiating a detailed transcription study, it was necessary to demonstrate that the the rho$^-$ strain genome was co-linear with the corresponding region in the wild type. To this end dD22 and 777-3A (wild type) mtDNA were digested with three different restriction enzymes. The restriction digest fragments of both strains were run in parallel on a 1.5% agarose-TEB gel. The separated fragments were transferred to a nitrocellulose filter and hybridised against nick translated ^{32}P-dD22 mtDNA (dD22, HindII 7kb) that contained the sequence between the cob leader and cob I5. Subsequent autoradiography allowed the visualisation of all fragments in the wild type encompassed by the dD22 mtDNA probe used. This approach allowed the direct comparison of the restriction site pattern of the mt DNA of rho$^-$ dD22 and the wild type 777-3A. Figure 12 shows the result of this experiment.

Figure 12 . Demonstration of the co-linearity of the wild type (777-3A) and the rho⁻(dD22). Autoradiogram of comparative restriction analysis of 777-3A and dD22 mtDNA. mtDNA restriction digests were electrophoresed through a 1.5 % TEB gel. After transfer to nitrocellulose the bound mtDNA was probed with a nick translated dD22 HindII 7 kb fragment.

It can be seen that the mt DNA restriction digest pattern of the rho⁻ strain dD22 and the wild type strain 777-3A are identical for the three restriction enzymes used. From this it

can be concluded that the mtDNA segment retained by the rho$^-$ dD22 contains no internal deletions, insertions or other such rearrangements as detectable at the level of restriction analysis.

3.2.4. Alignment of dD22 restriction enzyme sites with published data

The above analysis demonstrated the restriction site homology between the dD22 and 777-3A. By aligning the position of restriction sites defined by sequence analysis to the dD22 genome a restriction site map of dD22 could be constructed (fig. 13). The exact start and end position (junction) of the dD22 repeat unit was uncertain and the restriction enzyme sites around the junction have not been indicated. This map proved to be of great use in the isolation of DNA probes for transcript analysis.

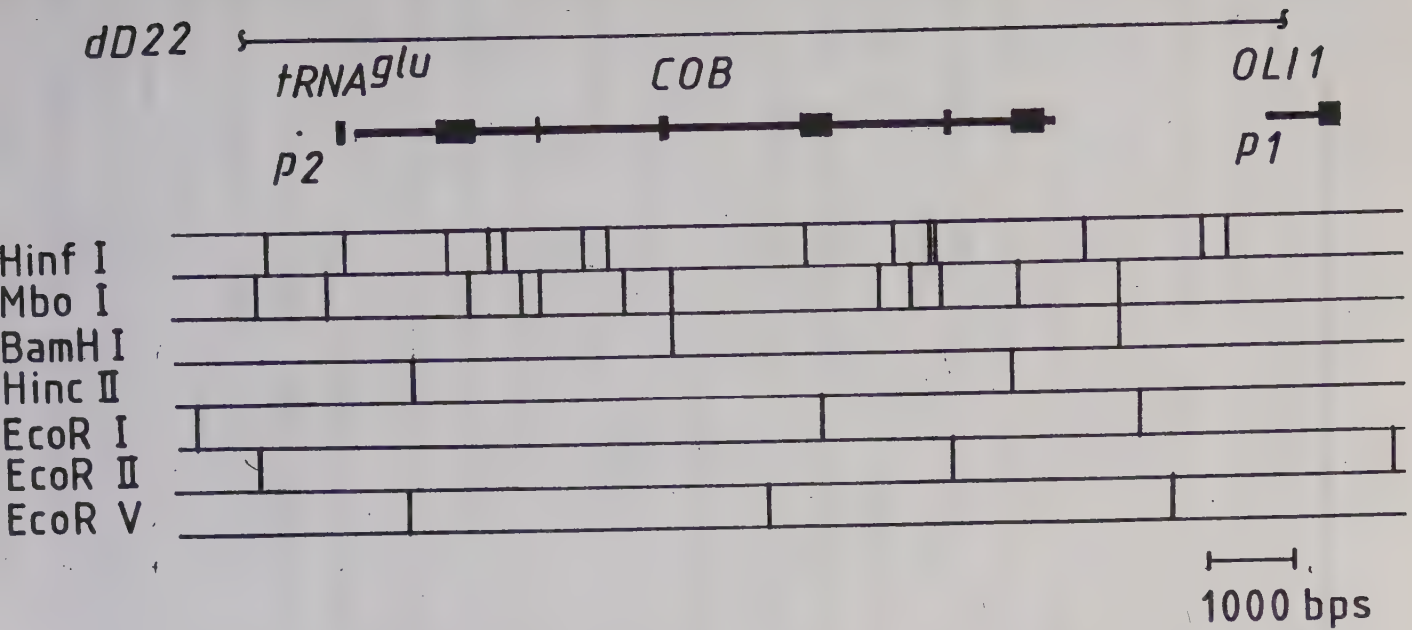

Fig. 13. Restriction map of the region encompassed by the rho⁻ dD22 showing the approximate position of the junction (J) and the promoters P1 (oli1) and P2 ($tRNA_{glu}$- cob). B1-B2 represent exon sequences. This map was compiled from the following data: 1 = restriction map of BC5 (section III.1. this thesis), 2 = Bonitz et al., 1982, 3 = Lazowska et al., 1980, 4 = Lazowska et al., 1980, 5 = Nobrega and Tzagoloff, 1980, 6 = Tzagoloff et al., 1980.

3.3. Demonstration of the functional intactness of dD22: reconstitution of a wild type genome

The above experiment was designed to demonstrate the intactness of the cob region retained by the rho⁻ strain dD22. However, the experiment was limited in its ability to detect minor differences such as deleterious base deletions or additions. Such minor changes could result in any one of a number of aberrant events such as incorrect transcription or processing. An alternative approach was applied with the aim of demonstrating the functional intactness of the cob region retained in the rho⁻ strain dD22. Use was made of the cob mit⁻ deletion mutant M9391. This mutant had been genetically characterised as being deleted for the entire cob region (R. Schweyen, personal communication). The rho⁻ strain dD22 was crossed with the mit⁻ deletion mutant M9391 (fig. 14) on glucose-YE medium. Subsequent selection for respiratory competent cells was performed on glycerol-YE. medium. Control crosses using oxi3 mit⁻ strains were also performed. After transfer to glycerol medium respiratory competent recombinant colonies were observed. The fact that dD22 was able to recombine with the cob deletion mutant M9391, to produce respiratory competent cells demonstrated that the sequences retained by dD22 represented a functionally intact segment of the wild type genome. Thus, any differences, if present, between the cob gene retained by dD22 and the cob gene present in the wild type genome were of no functional significance. The ability of the reconstituted wild type to grow on glycerol demonstrated that the dD22 genome contained an intact cob gene. This

reconstitution event presented several interesting possibilities: 1) did the insertion event involve any silent rearrangement of the cob gene ?, 2) had the dD22 cob gene been inserted at the site of the deleted cob gene in M9391 ?

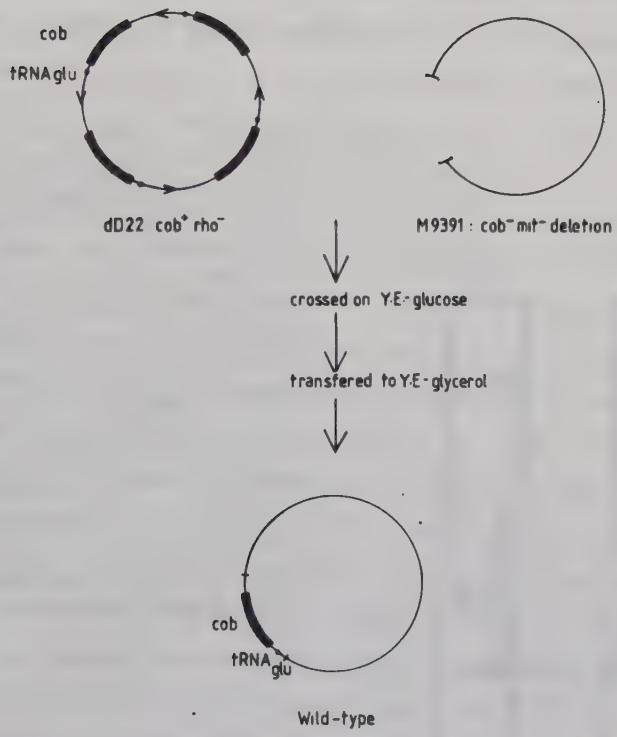

Fig. 14. Demonstration of the functional intactness of the cob gene retained by dD22. Schematic representation of the genetic strategy used to reconstruct a wild type genome using the rho− dD22 and the mit− deletion M9391.

3.4. Transcript analysis of the reconstituted wild type

Northern analysis of the cob transcripts from the reconstituted wild type revealed that it produced an 18S transcript identical in mobility to the putative cob mRNA (fig. 15). Difference in the quantities of pre-mRNAs were evident, and are probably a result of an alteration in the nuclear genome during the mating of the two strains or slight differences in the growth phase of the two strains at the time of harvesting.

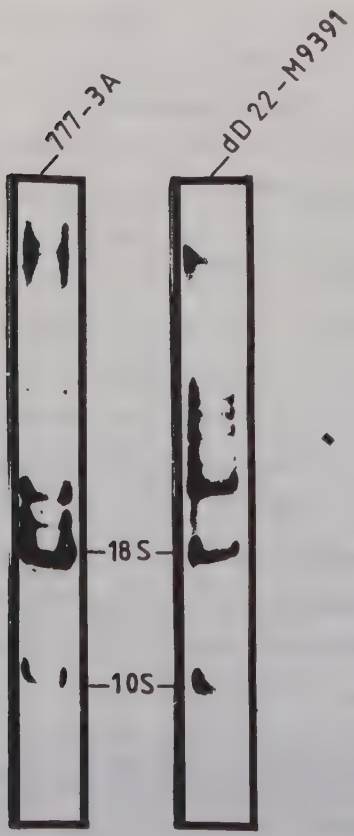

Fig.15. Comparative Northern analysis of the reconstituted wild type (dD22-M9391) and the wild type (777-3A). mtRNA from the reconstituted wild type (dD22-M9391) and the wild type (777-3A) was glyoxalated, electrophoresed on a 1.5 % agarose - 30 mM PO_4 gel and transfered to nitrocellulose (Thomas et al., 1980) The filter bound RNA was probed with a nick translated cob (dD22 x Hinc II 7kb) fragment. The additional pre-mRNAs seen in the dD22-M9391 mtRNA preparation are probably a consequence of different growth phase conditions at the time of harvesting or due to an alteration in the nuclear genome during cell fusion.

3.5. Direction of transcription in dD22

The mitochondria of yeast rho$^-$ strains lack many of the components present in the mitochondria of wild type strains. Their absence could conceivably affect the fidelity of transcription in such mitochondria. It was therefore considered necessary to compare the direction of transcription in the wild type strain 777-3A and in the rho$^-$ strain dD22. Since the polymerisation of ribonucleotides proceeds in a 5' to 3' direction, transcription in a given direction will be specific to one particular strand. Thus, to determine the direction of transcription it suffices to determine the strand origin of the transcribed RNA. This can be done by hybridising end labelled single stranded DNA to filter bound RNA. For this purpose mtDNA was isolated from a rho$^-$ strain retaining intron 5 and exon 6 of the cob region (MM1A7, Grosch et al., 1981). A Hpa II - Bgl II 214 bp fragment was isolated and 3' end labelled using (alpha^{32}P)dGTP and (alpha^{32}P)dCTP and the Klenow fragment of E. coli DNA polymerase. The labelled strands were denatured and separated on a 6% 30:1 bis-acrylamide gel. The ^{32}P labelled single strands were separately eluted from the gel and hybridised to filter bound mtRNA from dD22 and 777-3A. Figure 16 shows the autoradiogram obtained.

Fig. 16. Comparison of the strand origin of cob wild type (777-3A) and rho⁻ mtRNA (dD22). End labelled + and - strands of a cob exon 6 probe (rho⁻ MM1A7 x HpaII-BglII, 214 bps) were hybridised against filter bound mtRNA of dD22 and 777-3A. Only + strand RNA was detected, the faint signal seen using the - strand probe was due to the presence of residual amounts of mtDNA in the RNA preparation.

Using the end labelled strand complementary to the coding strand a signal was seen for 18S in the wild type and 32S and 20S in the rho⁻ strain. Using the strand complementary to the non-coding strand no hybridisation against any of the wild type or rho⁻ transcripts could be detected. This conclusively

demonstrated that the cob region of the rho⁻ strain dD22 was transcribed from the same strand and therefore in the same direction as the wild type.

3.6. Nuclear encoded intragenic processing of the cob gene

The maturase model of mitochondrial RNA processing predicts that certain mitochondrial intron sequences encode proteins essential for splicing of the pre-mRNA (Lasowska et al., 1980,). Intron mutations not only block the removal of the mutated intron but also of downstream introns. This polar effect is a consequence of the requirement of intron encoded polypeptides for the removal of certain mitochondrial introns. In the case of cob intron 2 the polypeptide encoded by this intron is apparently required for removal of intron 2 itself. Intron 1 does not encode any such polypeptide but mutations blocking its removal will also prevent the removal of introns 2, 3 and 4 (Schmelzer et al., 1981, Lazowska et al., 1980, Van Ommen et al., 1980). This is because translation of the intron 2 polypeptide is initiated at the AUG initiation codon of cob exon 1. Removal of intron 1 brings the exon 1 sequences in phase with the open reading frame of intron 2. Translation of exon 1, exon 2 and intron 2 sequences produces the polypeptide required to remove intron 2. Removal of intron 3 occurs in a similar fashion such that removal of intron 3 is dependent on removal of intron 2. The intron encoded proteins have been termed 'maturases'. Although not proven, it is implicit that the maturases are translated on mitochondrial ribosomes. This follows from the

observed occurrence in the intron reading frames of UGA codons whose recognition as tryptophan codons is unique to the mitochondrial genetic code (Barrell et al., 1979 and Macino et al., 1979).

3.7. Transcript analysis of a *cob* rho$^-$ strain

In order to determine the extent to which a functional system of mitochondrial protein synthesis is required for the maturation of apocytochrome b messenger RNA, the transcripts present in the rho$^-$ strain dD22 were analysed and compared to those sequences normally present in the wild type. Since rho$^-$ mitochondria lack a functional translational apparatus, it follows that any RNA processing of rho$^-$ mitochondrial transcripts must be the result of nuclear encoded and cytoplasmically translated proteins. It should be said that this argumentation is based upon the dogma that RNA is not exchanged between the cytoplasm and the mitochondrial matrix (Dujon, 1981).

Figure 17 shows an ethidium bromide stained gel of wild type (777-3A) and *cob* rho$^-$ (dD22) mtRNA. It can be seen that in comparison to the wild type the *cob* rho$^-$ strain lacks the two most prominent bands known to correspond to the two mitochondrial ribosomal subunits 21S and 15S. Clearly visible in the rho$^-$ mtRNA are five transcripts of 32S, 20S, 14S, 10S and 9S. Subsequent experiments were performed to characterise these transcripts in more detail.

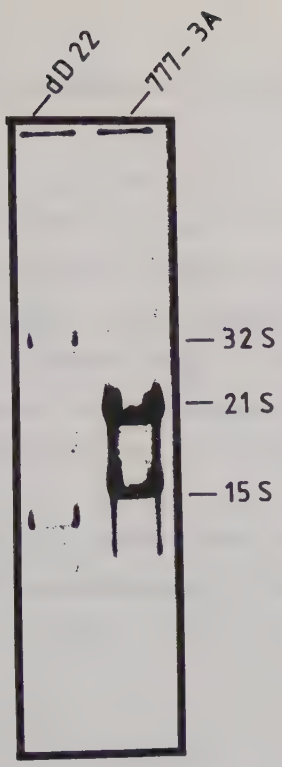

Figure 17. Comparison of wild type (777 - 3A) and rho$^-$ (dD22) mtRNA. Wild type (777-3A) and a cob rho$^-$ (dD22) mtRNA after glyoxalation and electrophoresis through a 30mM PO_4 1.5 % agarose gel and staining with ethidium bromide.

3.7.1. Northern analysis of dD22 mtRNA: comparison with intron mit$^-$ strains

Mitochondrial RNA was electrophoresed, bound to nitrocellulose and probed with a nick translated restriction fragment containing most of the cob gene (dD22 x HindII 7kb). To assist in the characterisation of the dD22 transcripts two mit$^-$ strains, M1301 and M561 were used as comparisons. M1301 bears a point mutation in intron 1 which blocks the removal of this intron and introns 2, 3 and 4 (the polar effect described above). M561 bears a point mutation in intron 2 which blocks removal of this intron and introns 3 and 4. Autoradiography of

the hybridised blot revealed the following (see fig. 18) :

777-3A (wild type, long form):

An 18S mRNA and several higher transcripts were detected in the wild type. Such higher transcripts have been previously characterised as intron bearing precursors of the cob 18S mRNA (Schmelzer et al., 1981). In addition a signal was also seen for a 10S transcript which is known to represent excised intron 1.

D273-10B(wild type, short form):

In contrast to 777-3A this strain lacks introns 1, 2 and 3 of the cob gene. Correspondingly this strain produced an 18S mRNA but did not produce a 10S signal confirming the identity of the 10S transcript as excised intron 1.

M1301 (mit^-):

The strain M1301 was seen to produce a 34S species. Accumulation of this cob transcript is due to a single base deletion in intron 1 which blocks excision of this intron (Schmelzer et al, 1981.). This block also prevents the excision of introns 2,3 and 4 although the excision of intron 5 is apparently unaffected.

M561 (mit^-):

The strain M561 was seen to accumulate a 32S. M561 has been previously characterised as being able to excise intron 1 and 5 but unable to remove introns 2,3 and 4, resulting in accumulation of a 32S transcript (Schmelzer et al., 1981). The cob precursors of M561 and M1301 provided useful molecular weight markers in the characterisation of the 32S transcript

seen in dD22.

dD22 (cob rho$^-$):

In dD22 four clear signals corresponding to a 32S, 20S and a 10S species were detected. The 32S transcript was seen to be identical in mobility to the transcript detected in the mtRNA of the intron 2 mit$^-$ strain M561. The comigration of the transcripts seen in dD22 and M561 indicated that dD22 accumulated a higher transcript bearing introns 2,3 and 4 but lacking intron 1 and 5. In the same way a dD22 transcript was detected identical in mobility to both the 10S transcript of 777-3A (wild type) and M561.

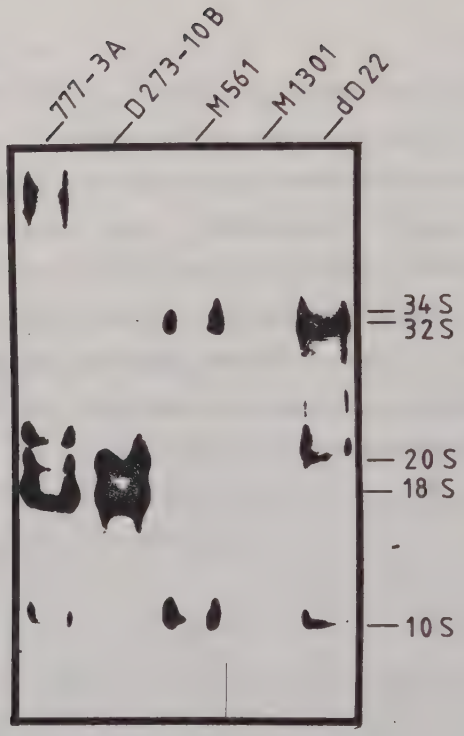

Fig. 18. Comparison of rho⁻ dD22 transcripts with those seen in wild type and two mit⁻ strains, M1301 (point mutation in intron 1) and M561 (point mutation in intron 2). Glyoxalated mRNA was electrophoresed and transfered to nitrocellulose according to Thomas, 1980. cob transcripts were detected by probing with nick translated dD22 x HindII 7 kb fragment. The wild type produces an 18S (the putative cob mRNA) a 10S (excised cob intron 1) and several high molecular weight transcripts which represent pre-mRNAs. The rho⁻ strain dD22 accumulates a 32S and a 10S transcript identical in mobility to the transcripts seen in the mit⁻ M561. It also accumulates a 20S transcript not seen in the wild type or mit⁻ strains.

The above data demonstrated that in the absence of a mitochondrial translation system the removal of introns 2, 3 and 4 was not possible. This is in agreement with the maturase model

of mitochondrial splicing as discussed above (section 3.6). In contrast the above data clearly demonstrate that in the absence of a mitochondrial translation system removal of intron 1 can occur. Hence, it appears likely that splicing of intron 1 is carried out by proteins translated on cytoplasmic ribosomes. Alternatively, the possibility that intron 1 is capable of self splicing must also be considered (Kruger et al., 1982).

3.7.2. Northern analysis of dD22 mtRNA: transcript mapping

The above experiment allowed an initial characterisation of the 32S and 10S transcripts seen in dD22. In order to determine the sequence composition of the 20S, 14S and 9S transcripts seen in the rho^- strain dD22 a series of sequence specific probes were isolated and hybridised against mtRNA blots of dD22 and 777-3A:

$tRNA_{glu}$- cob probe (dD22 x HindII(partial) 11 kb):
This probe hybridised against 32S, 20S, 14S and 10S transcripts in dD22. The identity of the 32S and 10S transcripts are described above. The 14S and 9S transcripts were not detected with the HindII 7 kb probe which contained sequences downstream of the cob leader and probably originate from sequences upstream of the cob leader (fig. 19-H).

Exon 1 probe (BA2 Mbo I):
As shown in figure 19-E the exon 1 probe hybridised to the dD22 32S transcript and the 18S wild type transcript. The dD22 20S, 14S and 10S transcripts were not detected with this probe.

Exon 6 - intron 5 probe (MM1A7, Hpa II - Hind II 900 bp fragment):

This probe hybridised against the wild type 18S and the dD22 32S and 20S. A previously uncharacterised transcript of about 9S was also seen (figure 19-G). Its precise identity was not determined. It could either represent excised intron 5 or a degradation product of the 3' side of the cob transcript. Such 3' degradation products have also been reported by Bonitz et al., 1982.

Intron 1 probe (Xba I - Bgl II 550 bp):

In both wild type and dD22 this probe hybridised against a 10S transcript (fig. 19-F). This result confirmed the above supposition that 10S represented excised intron 1.

cob leader probe (BC5 Hinf 1150):

This probe hybridised to the cob 18S mRNA in the wild type and the cob 32S (fig. 19-D) precursor mRNA in dD22.

The above experiments demonstrated that the rho$^-$ strain dD22 accumulated a cob transcript equal in sequence content and mobility to the 32S transcript seen in the mit$^-$ strain M561. This strain is known to be defective in the removal of intron 2, 3 and 4 of cob and capable of excising intron 1 and 5. Use of an intron 1 specific probe confirmed that the 10S seen in dD22 and M561 corresponded to excised intron 1. These data are interpreted as showing that the removal of intron 1 and probably of intron 5 is not dependent on the action of proteins

synthesised on mitochondrial ribosomes. The alternatives being either that they are spliced by factors imported from the cytoplasm or that the RNA present in the mitochondria of dD22 is capable of catalyzing the process of intron removal (Kruger et al., 1982).

Glutamic tRNA probe (BC5 Mbo 1 - Hinf 1 310 bp):
This probe hybridised against a 4S transcript in the wild type, assumed to represent the mature glutamic tRNA. In dD22 however, no mature tRNA could be detected, instead a 9S and a 14S transcript were observed (figure 19-C). These transcripts could represent tRNA precursors not visible in the wild type due to the rapidity of the tRNA processing steps concerned. Their presence in the rho^- strain was thought to be a consequence of the absence of the mitochondrial tRNA synthesis locus in the rho^- strain necessary for the processing of a tRNA precursor to its mature form (see discussion and below).

Upstream probe (BC5 MboI, 700 bp):
Using this probe no significant signals could be detected in the wild type. Against dD22 mtRNA only the 14S transcript could be detected (19-B).

The above data demonstrated that dD22 produces a 9S and a 14S species encompassed by the $tRNA_{glu}$ probe and that it does not synthesise a mature tRNA. Miller et al., (1984) have demonstrated that a mitochondrial locus located in the vicinity of the 15S rRNA is required for the 5' processing of mitochondrial pre-tRNAs. The inability of dD22 to produce a mature tRNA could therefore be explained by the absence of the

mitochondrial processing locus. Furthermore, Baldacci et al. (1984) have shown that mt-tRNAs in rho$^-$ strains have fully processed 3' ends. The 9S transcript accumulated by dD22 probably represents a pre-tRNA species with a 5'extension of about 450 bps and a normally processed 3' end. In support of this Christianson et al. (1983) have mapped the $tRNA_{glu}$ promoter to a point 450 bp upstream of the $tRNA_{glu}$ (section III. 2. 3.).

dD22 junction probe (EcoR V 3000 b.p.):
This probe extended from the 5' end of the cob leader across the dD22 junction. Signals were seen in dD22 against the 14S and 9S . In 777-3A hybridisation was seen against the 18S (cob mRNA) and a 12S transcript considered to represent the mRNA of the oli1 gene (fig.19-A). This indicated that dD22 contained part of the oli1 gene. The oli1 sequences retained by dD22 evidently includes the oli1 promoter which lies 631 upstream from the ATPase 9 AUG codon (Edwards et al., 1983). Taken together, the above data make it appear likely that the 14S results from initiation at the oli1 promoter followed by transcription across the dD22 junction . The exact position of its 3' terminus is uncertain but could well correspond to the 3' processing site of the $tRNA_{glu}$. The primary transcript, i.e. the precursor to the 14S probably extends from the oli1 promoter through both the tRNA and cob gene. Termination of transcription could presumably occur at the cob termination site. It should be pointed out that nothing is known about the termination of mitochondrial transcription, which opens up the alternative hypothesis that transcription initiated from the

oli1 promoter could proceed continuously around the rho⁻ genome, termination occurring non-specifically at any point on the genome (see discussion).

The 20S transcript detected with the cob probe appears to bear sequences transcribed only from the 3' side of the cob region. This conclusion is drawn from the observation that tRNA, leader, exon 1 and intron 1 probes did not hybridise against it, but the intron 5 - exon 6 probe did. There are two possible explanations for this observation: one is that the 20S transcript arises as a result of initiation within the cob gene, the other is that it is a degradation product, resulting from cleavage of the cob 32S precursor mRNA by which only the 3' half survives. On the basis of the above data it was not possible to distinguish between the two possibilities. The former explanation appears unlikely since in vitro capping experiments have demonstrated the absence of any gene internal sites of initiation (J. Edwards, pers. com.). Fig. 20 presents a schematic summary of the hybridisation data.

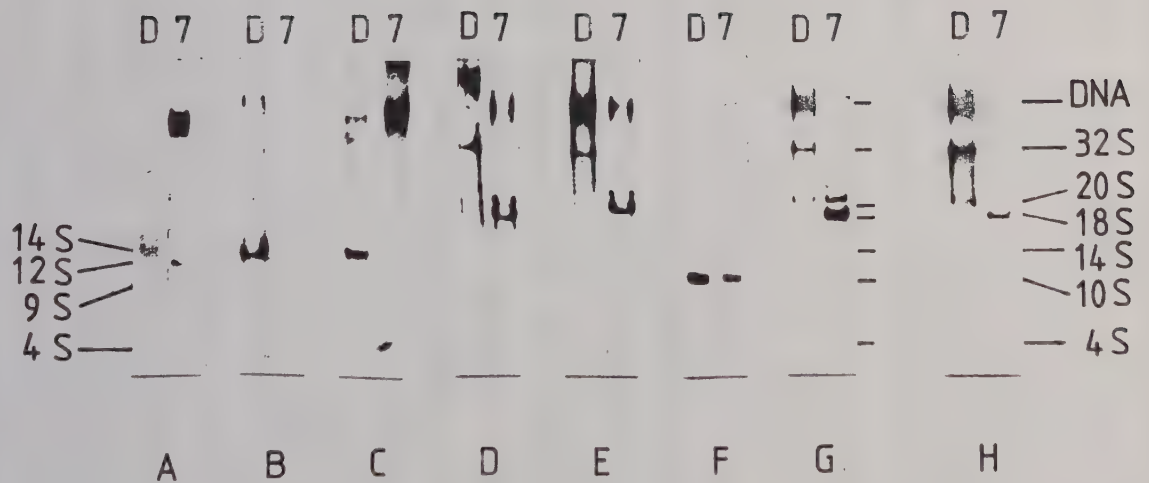

Fig. 19. Northern analysis of the rho⁻ dD22 compared to the wild type 777-3A. mtRNA was glyoxalated, electrophoresed in a 30 mM PO_4-agarose gel, transfered to nitrocellulose and hybridised against ^{32}P-DNA probes as described by Thomas (1980). Probes: A = dD22 x Eco RV - 3000 bps, B = BC5 x MboI 700, C = BC5 x HinfI-MboI 300 bps, D = BC5 x Hinf 1150, E = BA2 x MboI 500 bps, F = pDS1/CC14 x Xba1 x BglII 550 bp, G = MM1A7 x HpaII-HindII 900bps, H = dD22 x HindII(partial) 11kb.

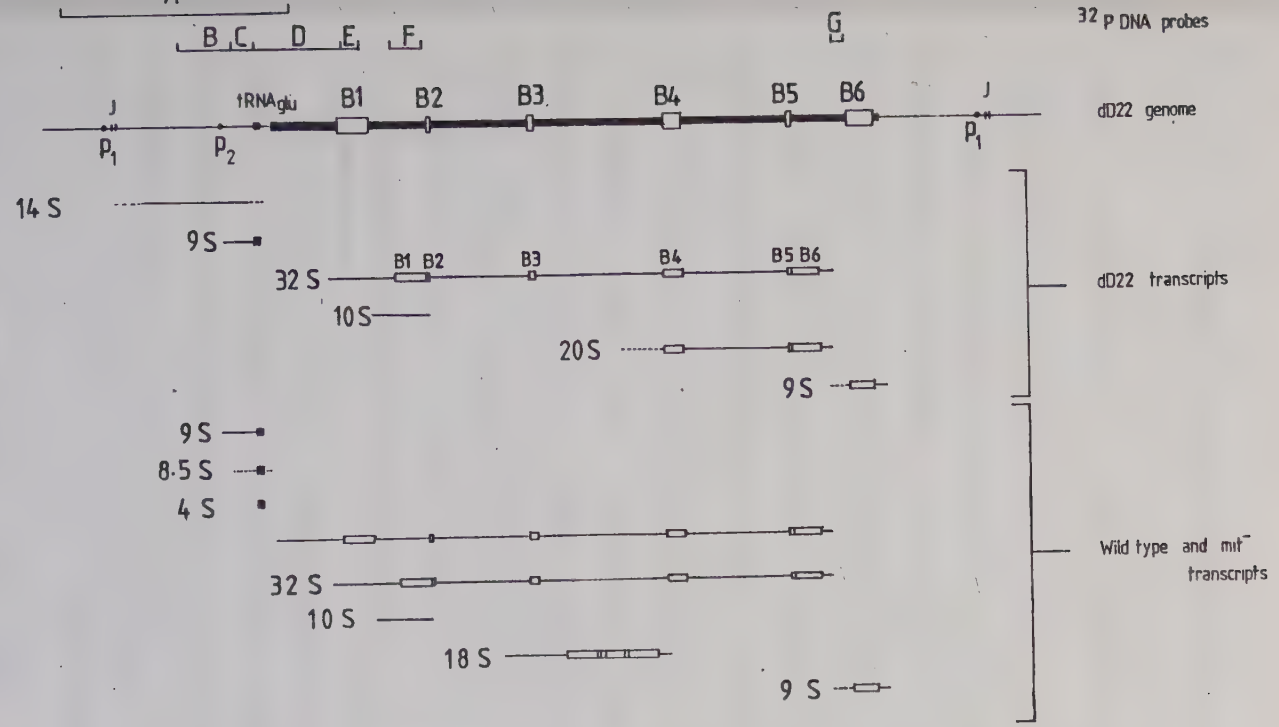

Fig. 20. Transcripts of the $tRNA_{glu}$- cob region in yeast mitochondria - Summary of hybridisation data. The data presented in sections 3.7.1 and 3.7.2 and figs. 20, 21 and 23 have allowed the construction of a transcript map of this region. Open white boxes represent exon sequences (B1-B2). Thick black lines represent leader and intron sequences. The open reading frames present in introns 2,3 and 4 are not shown. The ^{32}P-DNA probes are denoted A-G. P1 and P2 represent the oli1 and $tRNA_{glu}$- cob promoters respectively. J denotes the junction of rho$^-$ dD22.

3.7.3. Detection of tRNA precursors in mit$^-$ strains

The above data indicated that the glutamic tRNA was produced from a precursor transcript by way of at least two precursor species of 9S and 14S. Although both transcripts could be detected clearly in the rho$^-$ strain dD22 efforts to detect such precursors in wild type strains were unsuccessful. Hybridisation of a $tRNA_{glu}$ probe against a series of mit$^-$ strains bearing point mutations within the cob gene provided the surprising result that the mit$^-$ strain (M561) accumulated detectable quantities of tRNA containing transcripts of 9S and 8.5S, in addition to the mature 4S tRNA (fig 21). The 9S transcript co-migrating with the 9S transcript seen in the rho$^-$ strain dD22. The detection of these tRNA containing transcripts in strains bearing point mutations within cob introns could be explained either by some sort of interaction between the non-excised intron and the tRNA sequences or merely a consequence of the slower rate of growth exhibited by mit$^-$ strains in comparison to wild type strains on fermentable substrates. Whatever the reason for the accumulaion of the tRNA precursors in the mit$^-$ strain, there detection was of uppermost significance in understanding the events involved in the maturation of mitochondrial tRNAs. Although the 9S transcript is considered to represent a tRNA transcript bearing a 5' extension, the exact sequence of the 8.5S transcript is uncertain.

In dD22 minor signals were also seen against a series of high molecular weight transcripts of 23S, (4000 nucleotides) 27S

(6250 nucleotides) 34S (10,000 nucleotides) and further transcripts up to about 48S which would represent a molecule in the range of 100,000 nucleotides. The significance of these extremely long transcripts is unclear. The possibility that they represent cross hybridisation with cytoplasmic transcripts appears unlikely since they were only seen in the rho$^-$ strains and not in the wild type or mit$^-$ strains. The present view is that they represent oligo- or polymers of the rho$^-$ repeat units. This would mean that transcription would proceed through the rho$^-$ junction into and across the repeat unit of the adjacent repeat unit (see section IV.1.4.). In BC5 high levels of transcripts ranging from 7S to 16S were detected. Such transcripts were not detected in rho$^-$ dD22, mit$^-$ or wild type strains and appear to be specific to the rho$^-$ genome of BC5. Unlike dD22, BC5 does not contain a complete transcriptional unit (dD22 contains the co-transcribed region $tRNA_{glu}$- cob) and this could possibly result in abnormal transcription and/or processing of this region.

3.7.4. The import of RNA into the mitochondria

One of the central dogmas of mitochondrial biogenesis is that there is no exchange of polyribonucleotides across the mitochondrial membrane. The above data are in full support of this widely accepted view. Thus, analysis of the transcripts present in dD22 mitochondria reveals that all transcripts present, as visualised by ethidium bromide staining, can be detected by probing with nick translated mtDNA. No additional transcripts were detected which would appear to exclude the possibility of the import of RNA from the cytoplasm.

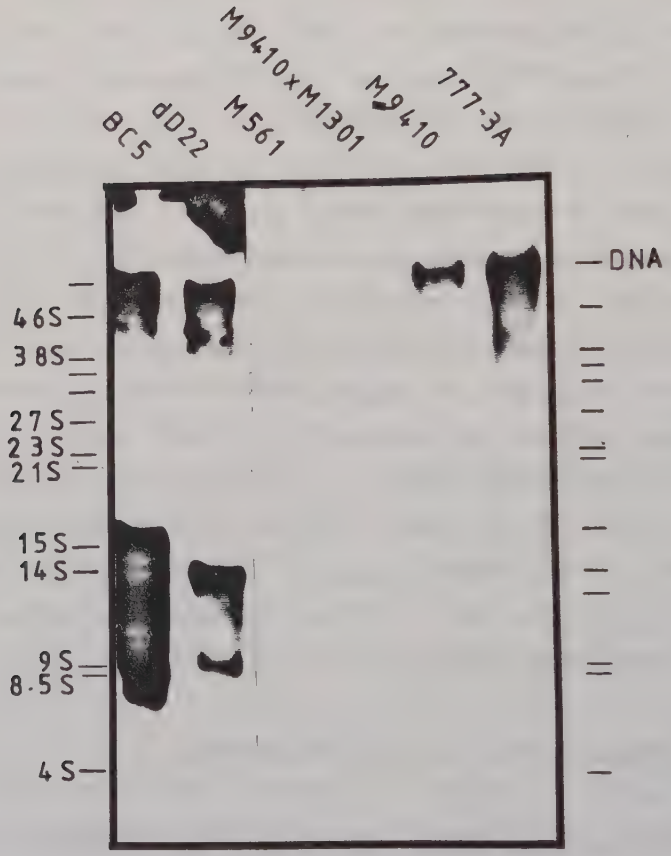

Figure 21. Detection of putative pre-tRNA$_{glu}$ transcripts in rho$^-$ and mit$^-$ strains. A 300 bp BC5 HinfI-MboI probe was hybridised against nitrocellulose bound mtRNA: mit$^-$ strains (M561, M9410xM1301, M9410), rho$^-$ strains (dD22 and BC5), wild type (777-3A).

4. Detection of mitochondrial initiation sites

4.1. *in vitro* capping of mitochondrial 5' di-phosphate termini

The 5' termini of yeast mitochondrial mRNAs differ from their nuclear counterparts by the lack of a 5' cap structure. In the nucleus the capping enzyme guanylyl transferase transfers a GMP molecule to a diphosphate end. Such diphosphate ends are characteristic of 5' ends that have arisen as the result of dephosphorylation of a triphosphate group produced by transcriptional initiation. This is in contrast to those ends 5' termini that have arisen as the result of a post transcriptional processing event which only bear a monophosphate end. Since yeast mtRNAs lack a cap structure a suitable assay for 5' ends corresponding to sites of transcriptional initiaton is to determine which termini can be capped *in vitro* . The capping enzyme (guanylyl transferase) catalyses the following reaction :

$$(\text{alpha-}^{32}\text{P})\text{GTP} + \text{ppN(pN)n} = \text{G(5')}^{32}\text{ppp(pN)n} + \text{PPi}$$

Experiments were conducted to determine the feasibility of this approach. Preliminary experiments using mitochondrial RNA isolated from wild type mitochondria produced three main signals. The strongest signal was seen with the 21S mitochondrial ribosomal subunit which indicates that the 21S rRNA bears a diphosphate end corresponding to the site of

transcriptional initiation. In contrast no signal was seen for the 15S rRNA, instead a weak signal was seen for a 15.5S species. This is interpretated as demonstrating that the 15S rRNA is produced by a 5' trimming event from a 15S precursor. The third signal migrated at about 12S and possibly corresponded to the mRNA for the ATPase subunit 9.

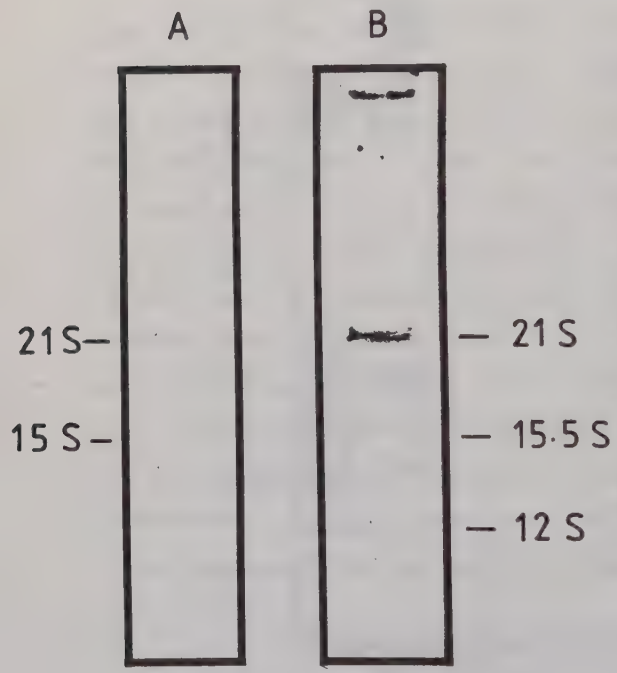

Fig. 22. Identification of transcriptional initiation sites in yeast mitochondria. Wild type (777-3A) mtRNA was incubated for 30 mins. at 37°C in 50 ul of 50mM Tris-HCl, pH 7.9, 1.25 mM $MgCl_2$, 2.0 mM DTT and 20 uCuries alpha-^{32}P GTP (3000 Ci/mmol) and 0.1 U guanylyltransferase. Non incorporated triphosphates were removed using a 2 ml column of bio-gel P40. The peak fraction was precipitated in ethanol, glyoxalated and electrophoresed as described by Thomas (1980).

5. Characterisation of a *cob* mit$^-$ deletion mutant

5.1 $tRNA_{glu}$ - *cob* spacer region

The mRNA for apo-cytochrome bears a 5' untranslated region (leader) of some 950 bp and is separated from the preceding gene for the glutamic tRNA by a spacer region of 115 bps (Bonitz et al., 1982). Although many other aspects of mitochondrial biogenesis are largely understood, the functional significance of the *cob* leader region and the 115bp $tRNA_{glu}$- *cob* spacer remain obscure.The isolation of a respiratory defective mutant deleted for some 900 bps encompassing most of the *cob* leader and part of the tRNA- *cob* spacer provided a unique opportunity to gain an insight into the role of this region in mitochondrial biogenesis.

5.2 Isolation and genetic mapping of M9410

The mitochondrial respiratory defective mutant M9410 was isolated by exposure of the wild type strain 777-3A to UV (R.J. Schweyen, personal communication). Subsequent genetic rho$^-$ deletion mapping revealed that the mutant locus mapped within the mitochondrial gene for cytochrome b. Fine recombination analysis using mit$^-$ strains and deletion mapping using rho$^-$ strains localised M9410 within a region spanning the 5' end of *cob* exon 1 and the gene for glutamic tRNA (R.J. Schweyen, personal communication).

5.3 Physical mapping of the M9410 deletion

Preliminary physical mapping indicated that M9410 was a deletion mutant (Schmelzer, 1981).Mitochondrial DNA was isolated from both the mit$^-$ M9410 and the wild type strain 777-3A. HinfI restriction enzyme digests of mtDNA from both strains were then electrophoresed in parallel and blotted onto nitrocellulose filters. The filter bound mtDNA was then probed with a nick translated Hinf 1150bp fragment, known to span the region between $tRNA_{glu}$ and cob . It was found that M9410 lacks the 1150 bp HinfI fragment present in 777-3A but contains a 280 bp fragment not present in 777-3A. All other fragments were common to both strains. These data demonstrated that M9410 was deleted for some 870 bp, between the two Hinf I sites which define the wild type 1150 bp fragment. This 1150 bp fragment is replaced by a 280 bp fragment in M9410 which has arisen by fusion of the DNA sequences flanking the deleted region.

5.4. Sequence analysis of the deletion junction site

For an accurate interpretation of the phenotype of M9410 a more precise definition of the deletion site was required. This was accomplished by sequencing both strands of the 280 bp Hinf I junction fragment (fig.23). Subsequent alignment with the wild type sequence (fig. 25) revealed that M9410 lacked 793bp normally present in the wild type. However, despite the nucleotide resolution afforded by the technique of DNA sequencing, the exact site of the deletion junction could not be determined. This is due to the fact that the junction is defined by a 32 bp sequence that exists as an almost perfect

direct repeat (R and R'in fig. 29) in the wild type. The differences between the two repeats occur as three mismatches between the two sequences, which divides the repeat unit into four sectors, a , b, c and d. In the junction fragment of M9410 this 32bp sequence apparently is composed of the 5' part of R and the 3' part of R'. It is possible to map the excision point of the deletion within sector C (13 bps long) some 70 bps upstream from the cob initiator codon AUG and 10 bps upstream of the 5' end of the cob mRNA.

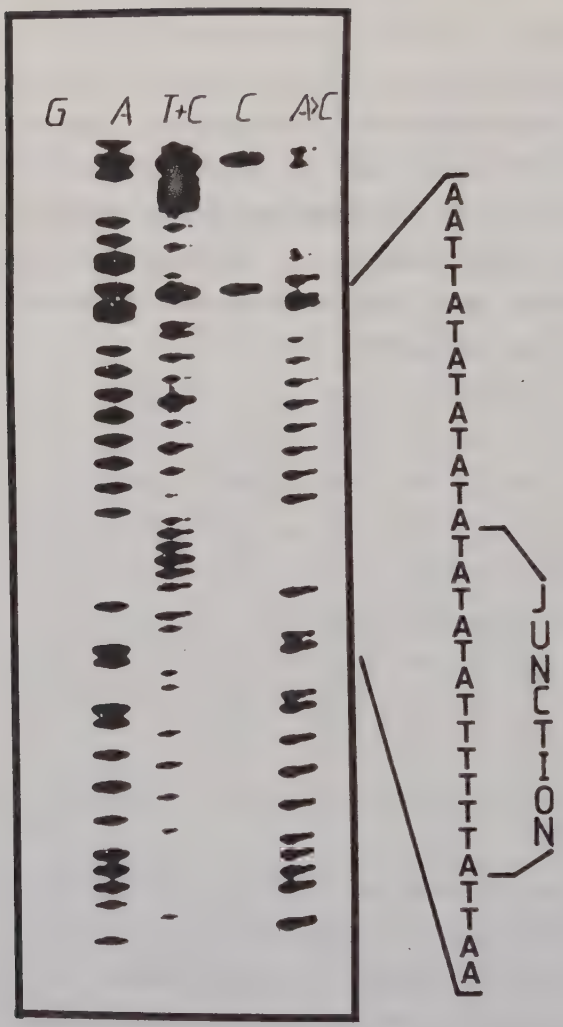

Fig. 23. Sequence analysis of the junction fragment of M9410. A 4.2kb HpaII fragment was isolated from M9410 mtDNA and redigested with HinfI. The HinfI 280bp junction fragment (see section 5.3.) was isolated and labelled at its 3' terminus with alpha ^{32}P dATP using Klenow polymerase. After strand separation on a 8% (30:1) TEB-acrylamide gel the end labelled strands were sequenced according to Maxam and Gilbert (1980).

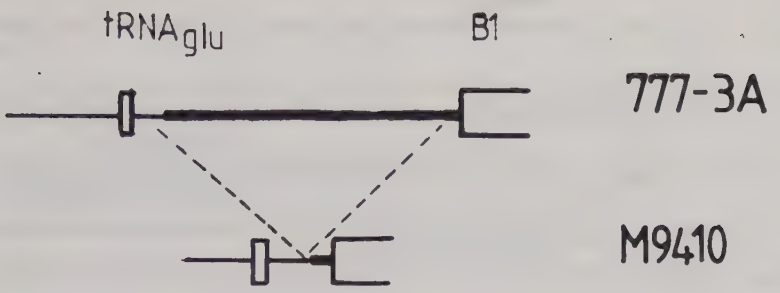

Fig. 24. Schematic representation of the position of the deletion as defined by sequence analysis. The deletion junction could be mapped to a point 76 bps (+/-6bps) upstream from the the cob fmetAUG (see text).

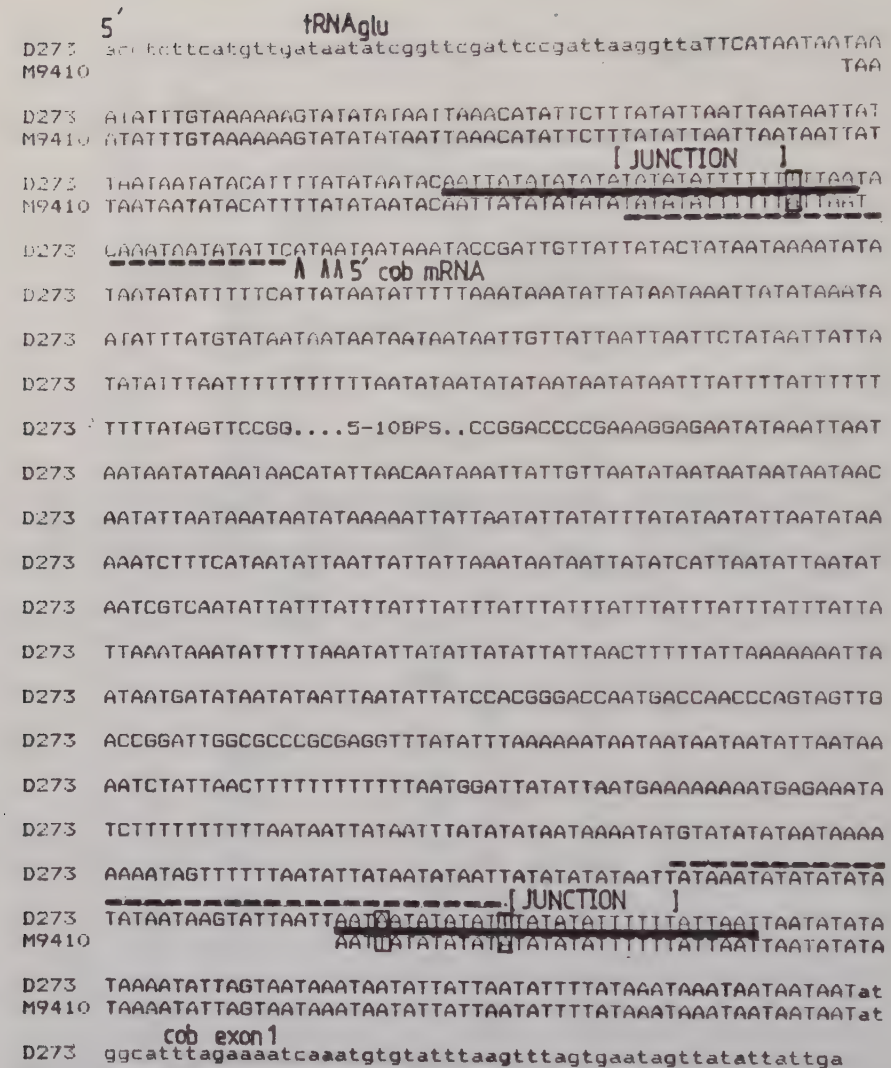

Fig. 25. Comparison of wild type (D273-10B, Bonitz et al., 1982) and the mit⁻ deletion (M9410) showing the position of the two 32 bp direct repeats (solid line). The mismatches, boxed nucleotides, define the deletion junction within a 13 bp sequence. Complementary inverted repeats are also present (dotted lines, see discussion).

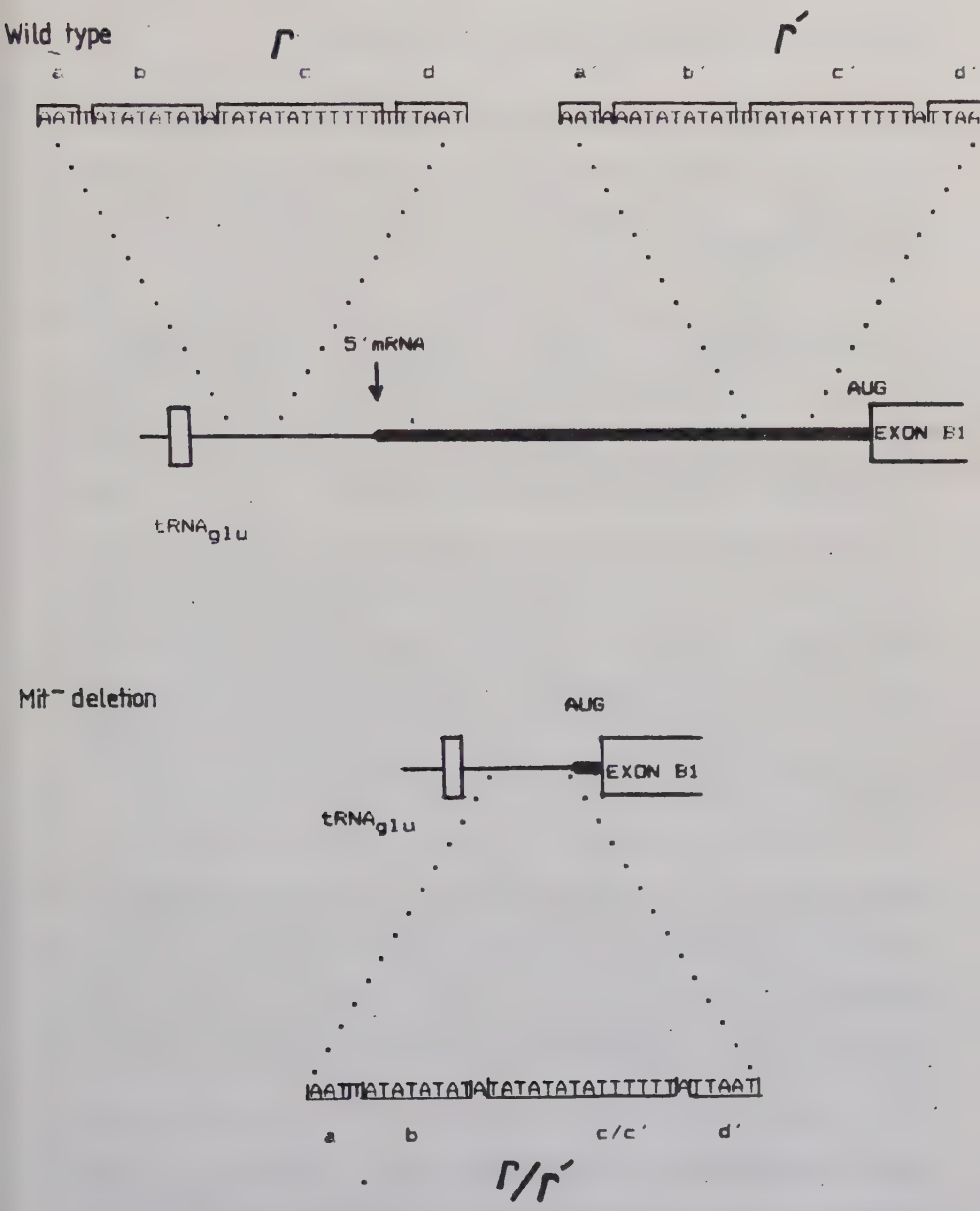

Fig. 26. Schematic represenation of the 32bp direct repeats that define the the junction of M9410. The 3 mismatches between the repeats help localise the deletion excision site (or junction) to sector c which lies about 70 bps 5' of the cob initiator codon.

5.5. Effect of deletion on expression of cob

Sequence analysis revealed that the deletion not only deleted most of the leader sequence but also some 10 bps of the $tRNA_{glu}$- cob spacer segment. Several consequences of this could be envisaged:

(1) Transcriptional - if the 5' end of the cob mRNA is identical with the site of transcriptional initiation then deletion of this site, as is the case in M9410, should prevent transcription of the remaining cob sequences.

(2) Post-transcriptional- if the 5' end of cob mRNA is not identical with the site of initiation, but occurs at some site upstream from the cob gene then the deletion should not interfere with transcriptional initiation. Of interest would then be to determine if the deletion acted at a post transcriptional level, e.g.transcript maturation.

(3) Translational- it is possible that the cob leader sequence acts as a target for ribosomes prior to the initiation of translation. Deletion of the cob leader sequence could conceivably block translation by eliminating a ribosomal binding site .

Clearly, inhibition of transcription will inhibit or prevent translation. For this reason the first experiments were designed to compare transcription in wild type and mutant mitochondria.

5.6. Transcript analysis of M9410

The first experiment compared the M9410 cob transcripts with those from the wild type strains. The method used was that of northern blot-hybridisation (Thomas, 1980). Mitochondrial RNA from M9410 and the wild type strain 777-3A was glyoxalated, electrophoresed and transferred to nitrocellulose filters. The filter bound RNA was then probed with a ^{32}P labelled DNA fragment containing most of the cob gene (dD22 x Hind II 7 kb fragment) Figure 27 shows the autoradiogram obtained from which it can be seen that the wild type produces an 18S mRNA and a 10S transcript. This transcript has been shown to correspond to a circularised excised cob intron 1 molecule. It appears to exist as an extremely stable species, probably due to its circular form (Halbreich et al., 1980). M9410 lacks the 18S mRNA but still produces a 10S transcript identical in mobility with the excised intron 10S transcript seen in the wild type. M9410 also produced an 11S transcript not seen in the wild type. The 10S transcript accounts for only 700 bps of the 10 kb cob gene. Other transcripts corresponding to the remaining portions of the gene were not detected. It was considered possible that the remaining transcripts if present were perhaps present at lower concentrations not detectable by the large cob probe used. In an attempt to detect low levels of cob transcripts smaller exon specific DNA fragments were isolated and used to probe the M9410 mtRNA (fig. 27):

Exon 1 probe - No M9410 transcripts complementary to this probe could be detected.

Intron 1 probe - This probe was used to verify that the 10 S RNA seen in M9410 was infact excised intron 1. Figure 27 shows that this was indeed the case whereby a 10S signal was seen both in the wild type and in M9410 when probed with a specific 10 S probe.

Exon 3 probe - As with the exon 1 probe, no RNA complementary to this exon 3 probe could be detected.

The failure to detect any cob transcripts using both the exon 1 and the exon 3 probes seemed at first to indicate that the deleted sequences included the cob transcriptional promoter. However, this was ruled out by the detection of the 10S intron 1 transcript which is transcribed in the wild type as part of the cob pre-mRNA (Schmelzer et al., 1981). These data show that in M9410 the cob gene is transcribed but that the deletion somehow leads to the rapid degradation of transcript. The presence of the intron 1 transcript is accounted for by the fact that it is excised and circularised immediately after transcription, circularisation presumably protecting it from the same fate as the remaining cob transcripts. The M9410 11S transcript seen using the cob probe was not detected using the the intron 1, exon 1 or exon 3 probes, this indicates that it originated from the 3' side of the cob gene. Its absence in the wild type implies that it is a consequence of the mutant

genotype of M9410. It could represent a 3' portion of the cob transcript that has avoided degradation.

5.7. Effect of leader deletion on $tRNA_{glu}$ maturation

The detection of a single site of initiation for the $tRNA_{glu}$ gene and cob indicates that they are co-transcribed (Christianson et al,. 1983). It was therefore of interest to determine the effect of the M9410 deletion on the transcription and maturation of the tRNA. Figure 27 shows that both the wild type and M9410 accumulate essentially equimolar amounts of a 4S transcript which is considered to represent the mature glutamic tRNA. The presence of $tRNA_{glu}$ and also of excised cob intron 1 were interpreted as indicating that the deletion did not interfere with the transcription of the $tRNA_{glu}$- cob transcriptional unit, but rather that it served to destabilise the cob pre-mRNA.

5.8. Possible involvement of the leader in intron processing

5.8.1. Construction of a leader deletion-intron 1 splice defect double mutant

The next step was to try and elucidate the cause of this instability. One possible explanation was that the leader sequence was needed to protect the cob transcript during the process of intron removal. To test this hypothesis a double mutant was constructed containing the M9410 leader deletion and a point mutation (M1301) in intron 1 known to block the excision of introns 1, 2, 3 and 4. This strain accumulates a 32S

transcript containing introns 1, 2, 3 and 4. Figure 28 shows the strategy used to construct the double mutant.

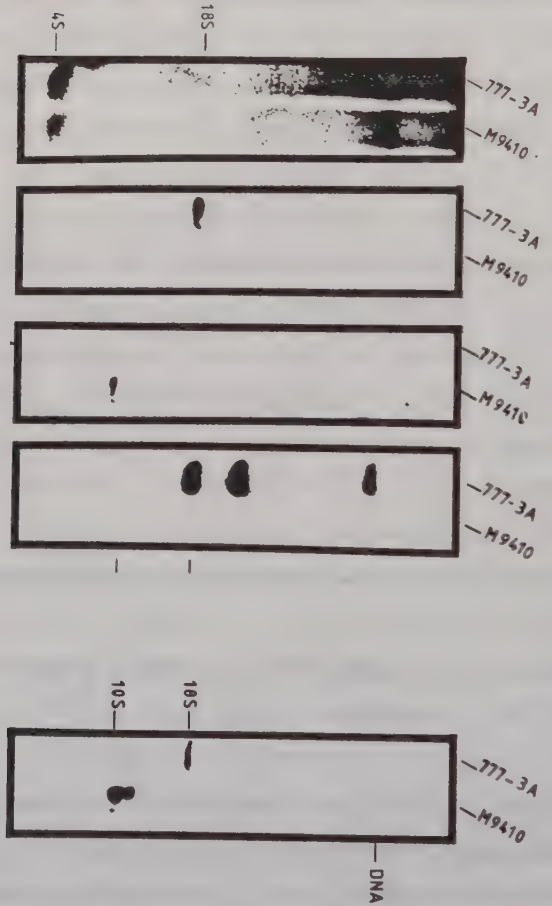

Fig. 27. Northern analysis of a mit⁻ deletion (M9410) affecting transcript stability. mtRNA from M9410 and 777-3A (wild type) was glyoxalated, electrophoresed in a 30 mM PO_4 agarose gel (Thomas, 1980) and bound to nitrocellulose. Probes: cob = dD22 x HindII 6kb, exon 1 = BC5 x HinfI-MboI 260 bps, intron 1 = pDS1/CC1+ (clone isolated from 777-3A) x XbaI-BglII 550 bps, exon 3 = A2A2 BamHI 700 bps.

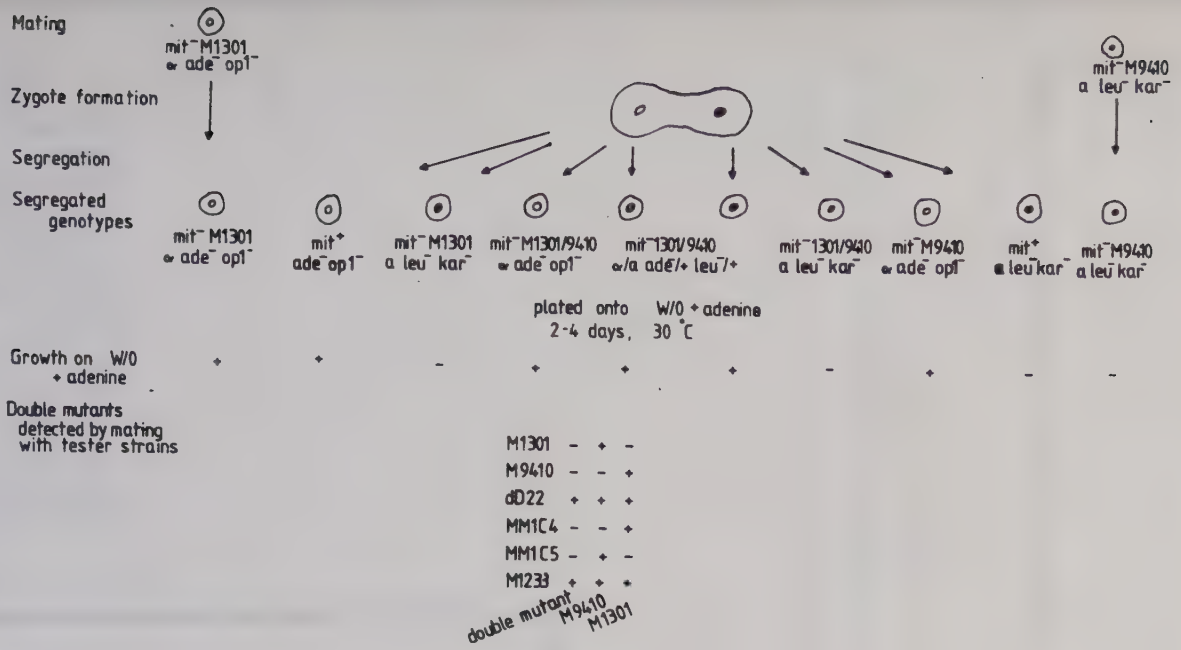

Fig. 28.Summary of the genetic strategy used to construct a mit⁻ double mutant of M9410 and M1301. Use was made of the kar⁻ genotype (Lancashire and Matoon, 1979) which hinders the process of nuclear fusion during zygote formation. The mitochondrial genotype is denoted as mit⁻ or mit⁺ (wild-type). The nuclear genotype (line below) shows the mating type alpha or a. Nuclear auxotrophies are designated by ade⁻ and leu⁻. op1 is a nuclear marker that is lethal in combination with rho⁻ mitochondria.

5.8.2. Transcript analysis of the leader-intron 1 double mutant

Mitochondrial RNA from the leader-intron 1 double mutant was compared to that of the single mit$^-$ strains. Figure 29 shows the result of this comparison.

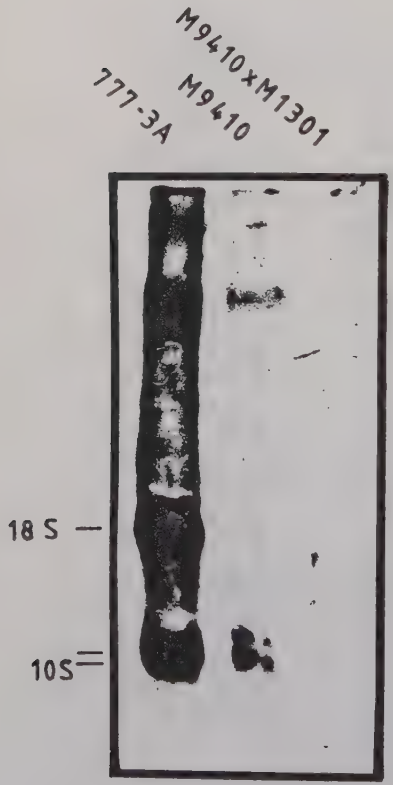

Fig. 29. To demonstrate the effect of combining the mit$^-$ deletion M9410 with a mit$^-$ blocked in intron excision (M1301). A ^{32}P *cob* probe was hybridised against nitrocellulose bound (Thomas, 1980) mtRNA from 777-3A (wild type) M9410, M9410xM1301, M1301.

It can be seen that both M9410 and M9410 x M1301 exhibit essentially the same transcript pattern (differences in the level of 10S between 777-3A and M9410 are due to differences in the total amount of mtRNA loaded onto the gel. Both strains exhibit low levels of aberrantly processed transcripts. However, the double mutant does not accumulate a 10S species seen in both wild type and the single mutant M9410. This is due to the point mutation introduced from the strain M1301. In conclusion it appears that the M9410 leader deletion exhibits epistacy over the M1301 intron 1 point mutation. The destabilising effect of the M9410 deletion does not appear to be connected with intron processing.

IV. Discussion

1.Mitochondrial transcription in the absence of mitochondrial translation

The mitochondrion contains the genes for the two rRNAs and a complete set of tRNAs. It has long been known that combined with other proteins imported from the cytosol they facilitate the translation of mitochondrial genes coding for various components of the respiratory chain, as well as intron encoded proteins believed to be involved in intron processing (Lazowska, 1980). However, little is known about the precise conditions necessary for accurate transcription of mitochondrial genes. Thus, although a nonanucleotide has been defined that is essential for transcription *in vitro*, this alone is not sufficient as yeast mitochondrial polymerase does not transcribe *Kluyveromyces* rRNA genes which also bear the same nonanucleotide (Osinga et al., 1982). It appears that other factors are necessary, either in the form of DNA sequences or in the form of diffusable factors involved in the modulation of transcription. It is of interest to note that the enzyme used in *in vitro* transcription has not been purified to homgeneity (Edwards et al., 1983) and that it may contain factors involved in the modulation of transcription and in the case of yeast mt polymerase specific for yeast mitochondrial promoters.

The occurrence of mitochondrial mutants lacking a mitochondrial translation system (rho^-) provides a unique opportunity to investigate the dependence of mitochondrial transcription and

RNA processing on mitochondrial translation. RNA processing in rho$^-$ mitochondria must be carried out either by proteins imported from the cytoplasm or by the RNA molecules themselves (Kruger et al., 1982 and Guerrier-Takada et al., 1983). To examine the contribution made by cytoplasmic factors, transcripts produced by wild type and cob rho$^-$ strains were compared in order to answer the following questions:

1) How does rho$^-$ transcription differ from wild type transcription?

i) Does transcription initiate at the same point as in the wild type?

ii) Are mitochondrially encoded proteins required for accurate transcription?

iii) Does the rho$^-$ junction influence transcription by acting either as a point of transcriptional initiation or termination?

iv) Is there any dosage effect on the rate of RNA processing, brought about by the increased copy number of the mtDNA segment?

2) Does RNA processing proceed as in the wild type?

i) What contribution does the cytosol make towards intragenic processing events, i.e. intron processing.

ii) What contribution does the cytosol make towards intergenic processing events i.e. 5' and 3' trimming events of pre-mRNAs and pre-tRNAs.

1.2. Direction of transcription

The mitochondrion of a rho$^-$ strain is devoid of many of the proteins normally present in the wild type. One of the consequences of this could be that the fidelity of mitochondrial transcription is reduced. A possible consequence of this is that the process of strand choice, as regards transcription might be affected. To investigate this possibility end labelled single stranded mtDNA corresponding to the anti-codon and codon strands was used to probe mtRNA. This revealed that only the anti-codon strand is transcribed in both rho$^-$ and wild type cells and that, therefore, rho$^-$ transcription was in the same direction as wild type transcription (fig. 16).

1.3 Fusion of the oli1 promoter to spacer DNA

During the formation of a rho$^-$ genome, sequences downstream of the retained segment are fused with sequences upstream of the retained segment. This fusion point is termed the rho$^-$ junction. In the case of the rho$^-$ dD22 the gene for apocytochrome b (cob) is retained in its entirety, but the downstream gene for ATPase subunit 6 is only partially present. It appears that the rho$^-$ junction lies immediately downstream from the oli1 promoter (fig. 20). Northern analysis of the transcripts of this region using a ^{32}P-DNA probe containing the dD22 junction detected a 14S transcript which extends from the junction, downstream, to a point adjacent to the 3' end of the $tRNA_{glu}$ gene. Although not proven, it appears that this 14S transcript arises by initiation at the

oli1 promoter. Its length and its hybridisation properties would indicate that transcription proceeds through the promoter for $tRNA_{glu}$. The 3' end of the 14S appears to be identical with the 3' end of the $tRNA_{glu}$ 4S transcript. It is likely that tRNA 3' trimming enzymes cleave both the pre-tRNA and the pre-14S transcripts. The actual point of transcriptional termination, is unknown. This point is discussed in the following section.

1.4. Continuous versus discrete transcription

Nothing is known about the factors or signals involved in the termination of mitochondrial transcription. In the rho^- dD22 the 3' end of the 14S transcript could be mapped to a point near the 3' end of the glutamic tRNA. This was not necessarily identical with the point of transcriptional termination. Transcription could well continue downstream into and through the cob gene, the point of termination perhaps being identical to that of the cob terminator, if such a sequence exists. An alternative is that transcription is not terminated, at any specific point, and proceeds continuously around the rho^- mitochondrial genome. This point is of extreme interest with regards the situation in wild type mitochondria since some 13 sites of transcriptional initiation have been mapped on the yeast mitochondrial genome (Christianson et al., 1983). The absence of transcriptional termination signals would mean that the mitochondrial genome consists of a system of continuously overlapping transcription units. Evidence for such a mode of transcription was presented in section III.3.7.3., whereby a

series of extremely high molecular weight transcripts were detected with lengths several times larger than the repeat unit of the rho$^-$ itself. This could indicate that transcription proceeds around the rho$^-$ genome and that processing subsequently divides up the transcript. Thus, it appears that at least in rho$^-$ mitochondria transcriptional units are defined at their 3' terminus by processing sites and not by termination signals. Extended to the wild-type this would represent a similar situation to that seen in human mitochondria whereby the complete genome is transcribed and cleaved into its constituent gene transcripts (Ojala et al., 1981). In yeast mitochondria there are multiple promoters spread throughout the genome (Christianson et al., 1983). At least six of these appear to promote transcription of multi-gene transcription units (Christianson et al., 1983 and Osinga et al., 1984). It is possible that each promoter does not only allow transcription of the gene(s) immediatly downstream of it, but that transcription proceeds through downstream promoter sites further around the genome.

1.5. Intragenic processing: *cob* intron processing

In section III. 3.8.2. it was clearly demonstrated that mitochondrial intron removal and exon splicing can occur in the absence of mitochondrial translation. Infact, the excised intron (*cob* intron 1) could be detected as a stable entity, existing as a transcript, also visible in the wild type, with a mobility of 10S. Since this process occurs in rho$^-$ mitochondria, it must occur independent of mitochondrially encoded translation products. One could envisage two possible alternatives. The

first is that intron processing is carried out by factors imported from the cytosol. The second alternative is that the intron possesses the catalytic potential for its own removal. The phenomenon of RNA molecules possessing catalytic activity has been demonstrated in two cases: 1) the intron of the rRNA of Tetrahymena is capable of self-splicing (Kruger et al., 1982), i.e. rRNA incubated in the presence of GTP and in the complete absence of proteins will undergo intron processing. Moreover, under certain conditions the excision process will result in the circularisation of the intron. 2) The RNA moiety (M1 RNA) of Ribonuclease P is the catalytic subunit of the enzyme (Guerrier-Takada et al., 1983). Ribonuclease P is responsible for the maturation of the 5' termini of tRNA molecules. It consists of a protein and an RNA subunit. In an incubation buffer containing Mg^{2+} and spermidine the M1 RNA can cleave the 5' tail of pre-tRNA molecules.

1.6. Intragenic processing: cut no-splice events

One of the transcripts detected in the rho$^-$ dD22 was a 20S. It was mapped to the 3' side of the cob gene. This transcript could either have arisen as the result of initiation at a gene internal position or as the result of a single cleavage event which cleaved the cob pre-mRNA internally, at a point corresponding to the 5' end of the 20S transcript. The former possibility is considered unlikely in light of studies conducted in the lab of Dr. Rabinowitz which have shown that initiation does not occur at gene internal positions, even in rho$^-$ mitochondria (J. Edwards, pers. com.). The latter possibility is probably more likely, other examples of mis-cleavage of

mitochondrial transcripts are also known. However, this explanation presents another problem; for if the 20S transcript arose by such a mis-cleavage event then why is the corresponding 5' side of the molecule not seen? Perhaps it is, for some unknown reason, simply highly unstable and is rapidly degraded. This problem is rather difficult to solve, one possible approach being to determine whether it bears a di-phosphate 5' terminus by use of the capping assay (see section III. 4.). Di-phosphate 5' termini are characteristic of 5' ends which correspond to sites of transcriptional initiation. Although a positive result would indicate that gene internal initiation had occurred, a negative result would be by no means conclusive, since phosphatase activity could have brought about the removal of the 5' di-phosphate group.

1.7. Intergenic processing: 5' processing of the cob pre-mRNA

Comparison of the transcript pattern of the cob rho$^-$ strain dD22 with that of a mit$^-$ strain (M561) blocked in the excision of introns 2, 3 and 4 revealed that both strains accumulated a 32S transcript considered to represent a cob pre-mRNA. As defined by northern analysis, the 5' terminus of this transcript appears to be identical with that of the mature 18S cob mRNA. The 5' end of the cob mRNA is apparently not identical with that of the site of transcriptional initiation for the cob gene. The cob promoter is apparently identical with that of the preceding gene for the glutamic tRNA (Christianson et al., 1983). The 5' terminus of the cob mRNA and the 32S pre-mRNA seen in dD22 and M561 is produced by

cleavage of a higher precursor. That this cleavage event occurs in rho$^-$ mitochondria indicates that this event is not dependent on the action of mitochondrially encoded factors.

2. Yeast mitochondrial tRNA maturation

The yeast mitochondrial genome encodes a complete set of tRNAs. Physical mapping of these genes has revealed a rather irregular distribution throughout the mitochondrial genome. Thus, some tRNA genes are grouped together as tRNA clusters while others are associated with rRNA or protein encoding genes (fig. 2). Although in mammalian mitochondria tRNAs appear to punctuate the genomic transcript (Ojala et al., 1981), the functional significance of tRNA distribution in yeast mitochondria remains obscure.

2.1. $tRNA_{glu}$ transcript maturation

The gene for the glutamic tRNA is separated from the gene for apocytochrome b (i.e. the point corresponding to the 5' end of the cob mRNA) by some 115 bps (Bonitz et al., 1982). Experiments reported in this thesis have produced several interesting results relating to the transcription and subsequent processing of this region. Preliminary experiments using RNA isolated from wild type mitochondria revealed the presence of a 4S RNA transcript which represents the mature glutamic tRNA. Subsequent analysis of mtRNA isolated from a rho^- strain containing the tRNA- cob gene segment failed to detect a 4S species. Instead, a 9S RNA species was detected. This 9S species probably represents tRNA bearing a 5' extension. This follows

from the work of Miller et al., (1984). A mitochondrial tRNA synthesis locus (not present in the rho$^-$ dD22) was shown to be required for 5' trimming of mitochondrial tRNA precursors. The failure to detect this 9S transcript in the wild type strain is probably a consequence of the speed with which 5' trimming occurs in wild type cells.

2.2 The yeast mitochondrial tRNA synthesis locus

The product of the mt-tRNA synthesis locus has been shown to act in trans. Deletion mapping has limited the tRNA synthesis locus to a 780 base pair sequence of DNA located between the $tRNA_{fmet}$ and $tRNA_{pro}$ genes on yeast mitochondrial DNA (see fig. 2). S1 and cDNA analysis have precisely located this RNA to a 450 bp DNA segment which appears to encode an RNA transcript of 9S whose presence is correlated with the ability of rho$^-$ strains to produce mature tRNA (Miller et al., 1983).

Construction of a "minimal structure" of the 9S RNA has revealed certain structural features similar to the RNA component of RNase P. This enzyme is necessary for the processing of the 5' end tRNA gene transcripts and requires a specific RNA for its activity. Recently, the group of Altman (Guerrier-Takada et al., 1984) demonstrated that the RNA moiety of RNase P is the catalytic subunit (see 1.5.). It is conceivable that the 9S RNA involved in mitochondrial tRNA synthesis also possesses the catalytic properties necessary for RNA cleavage at the 5' end of mitochondrial tRNAs.

2.3. *cob* intron mutants affect $tRNA_{glu}$ maturation

Experiments originally designed to detect the presence of mature glutamic tRNA in various *cob* intron mit$^-$ strains revealed the presence of a further tRNA containing transcript. This transcript was slightly higher in electrophoretic mobility than the 9S and was designated 8.5S. This transcript could also represent a tRNA precursor. This transcript either represents a second stage in the 5' processing of the glutamic tRNA or a glutamic tRNA transcript with a fully processed 5' end and bearing a 3' extension. This would represent the first report of mitochondrial tRNA precursors bearing a 3' extension.

The mechanism by which *cob* intron point mutations slow down the rate of tRNA maturation is unclear. A trivial explanation would be that it was simply a consequence of the decreased growth rate of the mit$^-$ strains compared to the wild type strains. However, rho$^-$ strains also exhibit a slower growth rate than wild type strains and they do not accumulate an 8.5S transcript.

Alternatively, it could reflect a specific interaction between the *cob* transcript and the glutamic tRNA transcript. This explanation is made more likely in light of the existing evidence for the co-transcription of the glutamic tRNA and the adjacent gene for apocytochrome b (Christianson et al., 1984). Thus one could envisage that a mutation preventing intron removal could somehow affect the conformation of the entire transcript an thereby interfere with other processing events both upstream and downstream from it.

2.4. RNA processing signals

Having identified several of the putative precursors of mature glutamic tRNA and the cob mRNA, an attempt was made to identify the nature of the cleavage signals. Examination of the DNA sequence in the immediate vicinity of the cleavage sites revealed several interesting features. For instance the sequence UAUAUUCAUAAUAAUAAAUA occurs both at the 5' end of the cob mRNA and at the 3' end of glutamic tRNA (S. Gerbl, pers. com.) (Fig. 30). This sequence could very well represent a recognition site for an RNA endonuclease. An interesting explanation, as to the origin of these two direct repeats, is that they may represent the termini of an inserted element which brought about the separation of the 5' end of the cob gene from the 3' end of the tRNA gene .

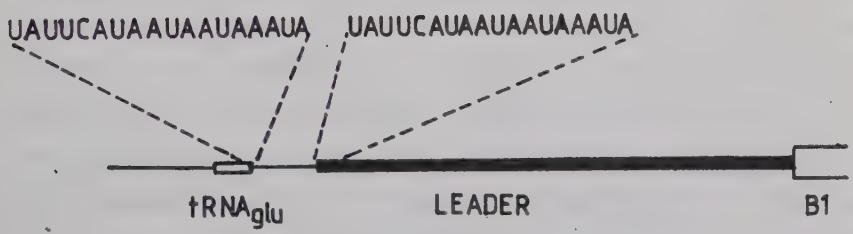

Fig. 30. Putative processing signals for the $tRNA_{glu}$ - cob co-transcript.

Other authors have implicated the role of much shorter sequences

in the process of intergenic processing. In the *var1* gene, for example, (Zassenhaus and Butow, 1984), the putative mRNA is generated from a precursor-RNA containing sequences of the ATPase subunits 9 and $tRNA_{ser}$ genes by cleavage at the heptanucleotide AAUAUAA. This non-descript sequence also occurs frequently at gene-internal positions, but only two of these are used to generate minor amounts of sub-genic RNAs with unknown function. How cleavage at the remaining sites is avoided is not clear , but factors like RNA secondary structure and ribosome-loading could well be involved. The latter explanation, however, would not explain the accurate processing observed in rho^- mitochondria which lack ribosomes. This heptanucleotide occurs nine times in the $tRNA_{glu}$- *cob* spacer region and its distribution failed to reveal any meaningful role for this heptanucleotide sequence in this region.

3.Effect of a mitochondrial deletion mutant on transcript stability

3.1. Mitochondrial intergenic spacers and 5' leaders

Mitochondrial genomes exhibit a remarkable degree of flexibility and variability both in genome and gene organisation. For instance in human mitochondria the gene for cytochrome b is butt-jointed to the preceding gene for $tRNA_{glu}$ whereas in yeast mitochondria not only are the two genes separated by a spacer of 115 bps but the cob mRNA also bears an untranslated 5' sequence of some 900 bps. The cob leader could be involved in some aspect of post-transcriptional or translational regulation. One popular notion is that the leader sequence serves as a target for ribosomes prior to the initiation of translation of the downstream coding sequences. Infact some authors have managed to visualise sequences supposedly representing ribosome binding sites similar to those seen in prokaryotes (Novitski et al., 1983 and Li et al., 1982). The above assumes that 5' untranslated sequences have a function. However, the possibility that it represents an optional sequence, that it has no significant function should not be excluded. As mentioned above human mitochondrial mRNAs lack any form of 5' untranslated sequences and are nevertheless present as stable translatable species. Infact recent studies (Denslow et al., 1983) have indicated that the 5' end of the human

mitochondrial cob mRNA is capable of forming a secondary structure which serves as a binding site for ribosomes.

3.2. A $tRNA_{glu}$- cob leader deletion mutant destabilises transcripts of the region

The isolation of a respiratory defective mutant deleted for most of the leader provided a unique opportunity to determine the role of the cob leader. Sequence analysis of this mutant (section III.5.4.) revealed that the mutant was deleted for some 950 bp from 70 bp 5' from the AUG to a site 10 bp 5' of the cob leader (fig. 28). Transcript analysis failed to detect any cob exon containing RNA: probes containing exon 1 or exon 3 sequences failed to detect any sort of signal, even after prolonged exposure of the autoradiogram. Infact, even the usual background smear present in most mtRNA preparations was absent. There are two alternative explanations for such an RNA phenotype: a) the deletion eliminated the cob promoter and thereby cob transcription, b) the deletion rendered the remaining cob RNA unstable and susceptible to rapid enzymatic degradation.

The former explanation seemed, at first, extremely likely in light of the knowledge that the deleted sequences included the 5' end of the cob mRNA which would have implied that the site of transcriptional initiation was identical with the 5' terminus of the cob mRNA. However, in yeast mitochondria initiation of RNA synthesis occurs in all cases within a short conserved sequence (Edwards et al., 1983, Tabak et al., 1983):

ATATAAGTA

-9 +1

The 5' end of the cob mRNA contains no such sequence. Furthermore in vitro capping and in vitro transcription experiments have failed to detect any initiation sites at the 5' end of the cob mRNA. It appears that the cob promoter lies some 500 bps upstream 5' of the gene for glutamic tRNA (Christianson et al., 1983, section III.2.4.). It thus appeared unlikely that the deleted sequences included the cob promoter. Nevertheless, these facts could be reconciled by assuming that the deleted region contained an enhancer element, normally required for transcriptional initiation at an upstream site. (Yaniv, 1982). This explanation would assume that deletion of the enhancer sequence in M9410 brought about the inactivation of the upstream promoter. However, this possibility was excluded by demonstrating that despite the absence of exon containing transcripts the cob gene was being transcribed at essentially wild type levels. This was revealed by the detection of wild type levels of cob intron 1. Detection of normal levels of this transcript indicated that the cob gene was transcribed but that the lesion somehow rendered the cob transcripts, excluding excised intron 1 susceptible to rapid degradation. The survival of excised intron 1 is explained as follows: firstly, removal of intron 1 of the cob gene appears to be an extremely rapid process, excision probably occurring immediately after transcription of intron 1. This is indicated by the finding that intron 1 sequences were only detected in the excised form as a 10S RNA species. Secondly, the excised intron appears to undergo

circularisation which renders it considerably more stable than the corresponding linear form (Halbreich et al., 1980). There still existed the remote possibility that the deletion had indeed blocked transcription from the cob promoter and that the transcription observed in the form of excised intron 1 was due to initiation at a secondary promoter immediately upstream of intron 1. This possibility was excluded by the detection of wild type levels of a transcript corresponding to $tRNA_{glu}$ (the present evidence indicates that $tRNA_{glu}$ and cob are part of the same transcriptional unit being transcribed from the same promoter which lies about 500 bps 5' of $tRNA_{glu}$) (Christianson et al., 1983). Hence, the cob -$tRNA_{glu}$ promoter appeared to be normally active and it was concluded that the affect of the deletion was at the level of transcript stability. Figure 31 summarises the above interpretation.

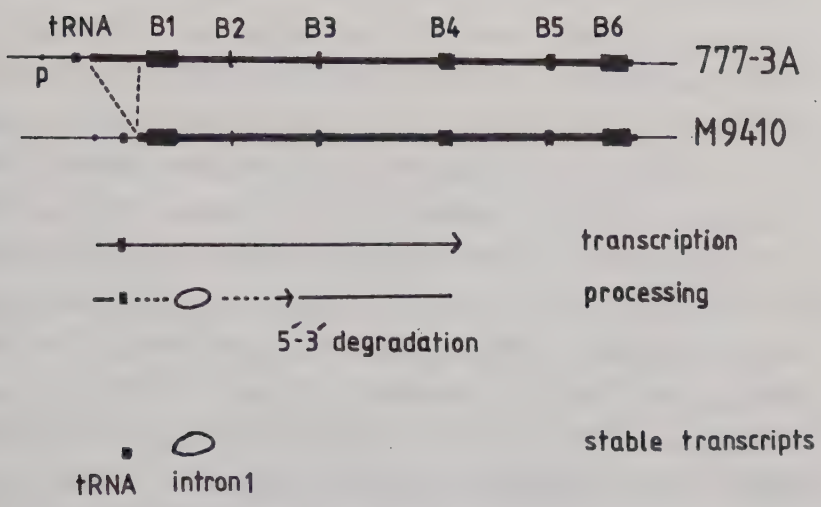

Figure 31. The effect of the M9410 deletion on RNA processing of the cob region. It is envisaged that the deletion destabilises the pre-mRNA such that it is degraded in a 5'-3' direction from a site corresponding to the 3' processing site of the glutamic tRNA.

3.3 Mechanism of transcript destabilisation

Sequence analysis of the deletion mutant (M9410) revealed that most of the leader been deleted, including the sequence corresponding to the 5' end of the mRNA had been deleted. It was therefore difficult to determine whether deletion of the leader sequence or deletion of the 5' end of the mRNA was the crucial factor responsible for the absence of cob exon RNA. The possibility that deletion of the leader sequence alone resulted in the susceptability of the cob RNA to enzymatic degradation assumes that one of the functions of the leader sequence is to

protect the downstream coding sequences from enzymatic degradation, perhaps by the formation of some sort of RNase resistant secondary structure. Another explanation is that it is merely the deletion of the processing site for the 5' end of the mRNA that destabilizes the cob RNA. This explanation assumes that correct 5' processing of the cob RNA is essential for the production of stable transcripts. It is possible that the normal 5' processing event also involves a base modification at the 5' end of the cob pre-mRNA which renders the RNA resistant to 5'-3' exonucleolytic degradation. The recently reported findings of Tzagoloff and his co-workers (Dieckman et al., 1983) are in good agreement with the above hypothesis: characterisation of a nuclear mutant (pet$^-$ CBP1) affecting maturation of the cob RNA reveals that it exhibits a similar phenotype to the mit$^-$ deletion mutant M9410 characterised in this thesis. Thus, the only transcript present in significant quantities is a 10S species corresponding to excised intron 1. A mitochondrial revertant of this pet$^-$ strain was also isolated and was shown to produce a cob mRNA bearing a leader of only 28 bps. This fact alone indicates that the cob leader sequence per se is not an essential part of the cob mRNA and that it can exist as a stable transcript without the full 900 nucleotides normally present. One could envisage that the original pet$^-$ mutation was brought about by the alteration of a factor involved in 5' processing. Presumably, in the mitochondrial revertant a sequence alteration occurred which allowed the altered processing factor to process the cob pre-mRNA at a point 28 bps 5' of the initiation codon AUG. This processing event like the original one at -950 bps apparently produced a cob pre-mRNA capable of fitting in to the RNA processing system.

3.4. Degradation of non- excised intron 1

To gain a further understanding of the mechanism by which the deletion destabilised the cob transcripts, a double mutant of M9410 and a cob intron 1 mutant was constructed. The intron 1 mutant (M1301) was blocked in the excision of intron 1 and accumulated a 33S transcript. Northern blot analysis of the cob transcripts of this double mutant revealed that it possessed exactly the same RNA phenotype as the M9410 single mutant except that it lacked the 10S transcript corresponding to excised intron 1 (a consequence of the M1301 mutation). This result demonstrated that the M9410 was epistatic over downstream mutations which normally cause the accumulation of high molecular weight transcripts. The fact that the non-excised intron was also degraded in the double mutant is further support that the presence 10S in M9410 is a consequence of its rapid excision from the cob pre-mRNA and not due to re-initiation between the site of the deletion and intron 1.

4. Mechanism of deletion formation

This dissertation has focused on the processes involved in yeast mitochondrial transcription. One approach involved the characterisation of a mit^- strain (M9410)deleted for some 900 bps. This deletion was induced by exposure to UV radiation. In this section the possible mechanism by which such a deletion could have arisen will be discussed. It is evident that the mechanism of induction of chromosome aberrations by UV radiation are complex and diverse. Nevertheless, the end-results, in the form of structural rearrangements are fairly uniform. In this section the DNA sequence of the excision site of M9410 has been used in an attempt to deduce the deletion mechanism.

In yeast mitochondria two forms of deletion can be characterised: (a) The straightforward deletions are represented by the mit^- deletion mutants, (b) the rho^- deletion mutants however are macrodeletions of the mitochondrial genome wherein the retained portion is amplified several times to produce a circular rho^- genome containing several to many copies of the retained segment. Taken together the rho^- and the mit^- deletions could be seen as representing reciprocal events in the process of mitochondrial deletion formation. In this way it is envisaged that the type of mutant strain detected depends on whether the deleted segment is retained (and later amplified) to form a rho^- or whether the remaining segment lacking the deleted sequences is reclosed and retained to form a mit^- deletion mutant.

The mit$^-$ deletion M9410 was found to be defined by two direct repeats of 32 bps containing 3 mismatches which divided the repeat unit into 3 subsectors a, b, c, and d. These mismatches allowed a precise localisation of the M9410 junction to the 13 bp subsector c.

4.1. Slipped mispairing model

The finding that the leader deletion of M9410 is defined by two direct repeats prompted the suggestion that "slipped mispairing" (Streisinger et al., 1966, Efstratiadis et al., 1980, Albertini et al., 1982) during DNA synthesis might have been involved in the formation of the leader deletion of M9410. Briefly, this model (fig. 32) assumes the presence of two direct repeats (shown as D1 and D2) in the vicinity of the replication fork. A downstream repeat is assumed to pair with the complementary upstream repeat of the opposite strand. This mispairing event is brought about by the slipping of the two DNA strands in the vicinity of the replication fork which results in the formation of single stranded loop whose formation is believed to be facilitated by the pairing of the two inverted repeats (shown as I and I' in fig. 32). The M9410 deletion junction could be localised to a 13 bp sub-section (c and c') of the 32 bp repeat unit. It is conceivable that strand slipping followed by mispairing between c and c' represents the mechanism by which the the M9410 deletion was formed. Sub-sectors c and c' are internally flanked by inverted repeats (see fig. 25) capable of pairing with one another. They may have facilitated deletion

formation in M9410 as mentioned above. Furthermore, sector c is surrounded by three other sub-sectors of the 32 bp repeat unit; it is possible that slipping and mispairing among these sequences may have preceded and facilitated the slip-mispairing event that resulted in deletion formation.

Evidence for the occurence of 'slipping' during DNA replication was also revealed by the detection of a DNA sequence strain polymorphism. This polymorphism was detected in the form of an insert of some 75 bps. Of interest was that the sequence immediately 3' of the insert was almost identical to the sequence of the insert itself. In addition this sequence was extremely thymidine rich (as regards the coding strands). Such sequences are particulary prone to deletion events, probably by a slipping mechanism as originally proposed by Streisinger (1966). Further evidence comes from the observations of Weiss-Brummer et al. (1983) who detected the following deletions and insertions when studying mutations and reversions in intron 4 of the cob gene:

wild type: GAGACTACACGCACACTAAAA

Deletion: GAGACTACGCACACTAAAA

Insertions: (a) GAGACTACGCGCACACTAAAA

(b) GAGACTACTACGCACACTAAAAAATAAA

(signifies repeat sequences)

According to the model of Streisinger (1966) such mutations may have been generated by slipped mispairing as depicted for insertion (b) below:

```
1.   GAGACTACGCACACTAAAA          new strand synthesis
     CTCTGATG

2.   GAGACTACGCACACTAAAA          slipped mispairing
     CT TG
      C A
      T-G

3.   GAGACTACGCACACTAAAA          completion of strand
     CT TGATGCGTGTGATTTT
      C A
      T-G

4.   GAGACTACTACGCACACTAAAA       replication of new strand
     CTCTGATGATGCGTGTGATTTT
```

In summary, three sorts of mitochondrial deletions (as classified by size) can be accounted for by the process of slipped mispairing. Thus although, other mechanisms can also account for the formation of the M9410 deletion (see below) only slipped mispairing can account for all three.

4.2. Deletion by non-homologous recombination

Alternatively the mechanism (for M9410) could have simply involved non-homologous recombination involving the formation of a coiled structure, stabilised by inter-duplex pairing between the two direct repeats (figure 33).

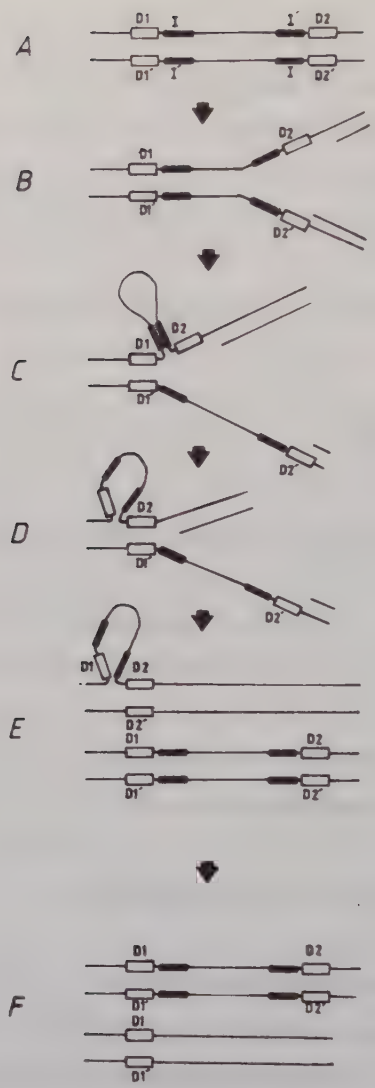

Fig. 32. The slipped-mispairing model of deletion formation (adapted from Albertini et al., 1982).

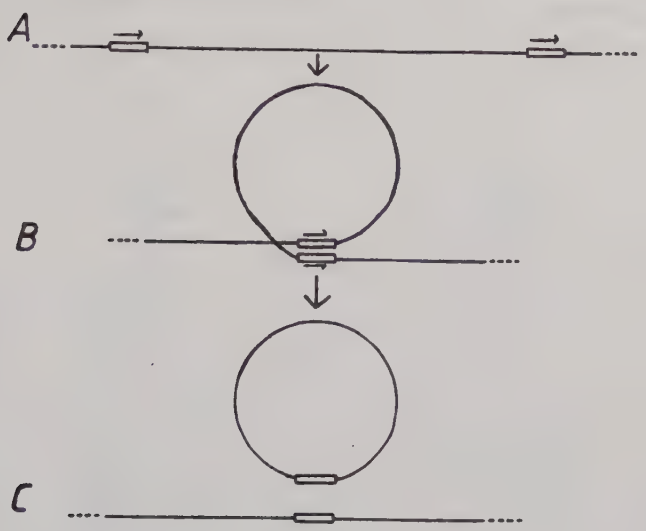

Fig. 33. Formation of a mit⁻ deletion by non-homologous recombination.

4.3. Deletion by formation of an intermediate hairpin structure

The two direct repeats that define the M9410 junction, though relatively large, were not necessarily involved in the recombination event. Closer examination of the sequences defining the deletion site in M9410 revealed the existence of inverted complementary sequences which could be paired to form the structures depicted in fig 34-a and b. In fig. 34-a the deletion junction is represented by the base of the stem. In fig. 34-b (which depicts a more stable structure than 34-a) the junction is within the stem. Such structures could be formed on

both strands in a cruciform manner and the deletion process could possibly occur enzymatically by cleavage at the base of the stem structure in a or within the stem in b. (fig. 35).

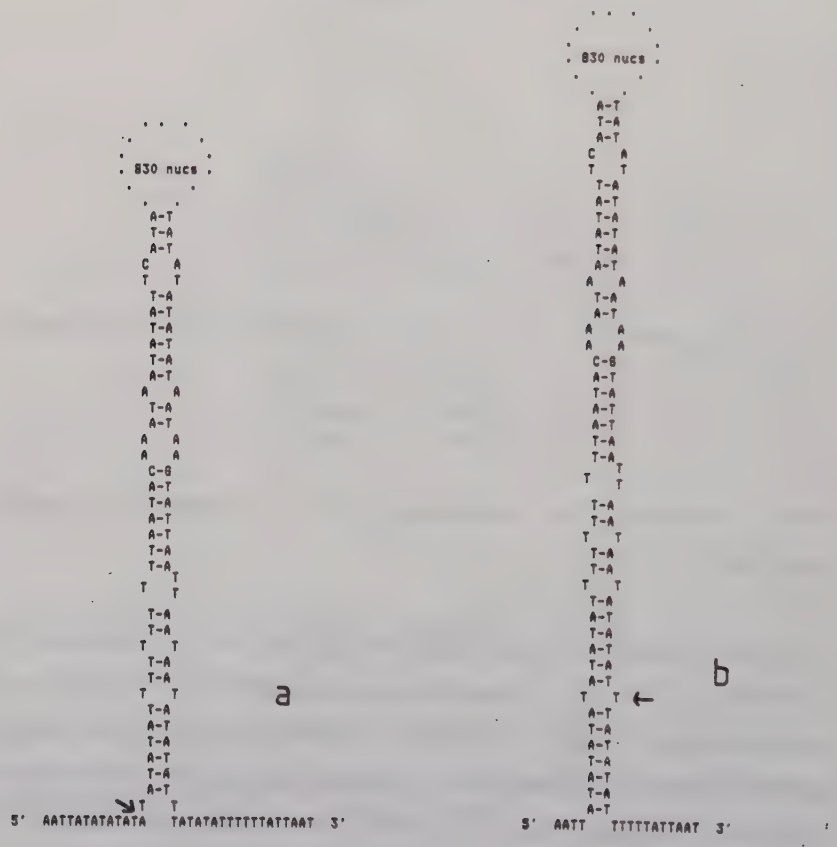

Fig.34 .Hairpin structures possibly involved in the formation of the deletion M9410. In structure (a) excision occurs at the base of the stem, in (b) it occurs at the first bulge.

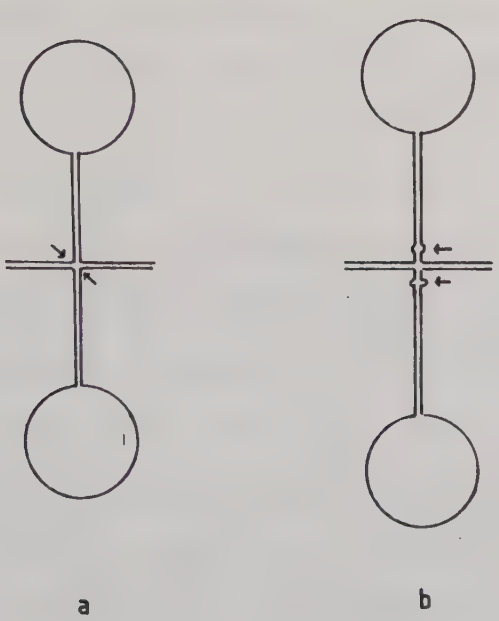

Fig. 35. Cruciform structures possibly involved the formation of the mit$^-$ deletion M9410.

4.4. Mitochondrial recombination: excision sequences involved in rho$^-$ formation

Mechanisms of mitochondrial recombination are poorly understood, however, all of the above models can be united by visualising the processes of mit$^-$ deletion and rho$^-$ formation as reciprocal events. This is particularly evident from fig. 33 whereby it could be envisaged that either a rho$^-$ or a mit$^-$ is formed depending on whether the excised loop or the remaining genome is retained. The above described models differ in the nature of the sequences involved in the recombination

event. The first two mechanisms implicated the involvement of direct repeats in the deletion event. The last model relied solely on the presence of inverted repeats. Other authors (Gaillard et al., 1980 and Bernardi et al., 1980) have also implicated the involvement of direct repeats in the excision event. Analysis of the repeat units of the mitochondrial genomes from several spontaneous rho^- strains (Faugeron-Fonty et al., 1979) have shown that these rho^- genomes fall into two classes: (i) repeat units excised at GC clusters which appear to be more frequent (ii) repeat units excised elsewhere, most probably in the AT spacers. Sequence analysis of the repeat unit of two class (ii) rho^- has shown that they are excised at two pairs of direct repeats located in the AT spacers (Gaillard et al., 1980, Bernardi et al., 1980). In both cases, the direct repeats were 13 nucleotides long, with one mismatch in one case. Analysis of the repeat unit of a class (i) rho^- revealed that the excision sequences were represented by 23 base pair repeats (instead of 13 in class (ii). Furthermore, the excision sequences of class (i) repeats were 80% GC, compared to 100% AT in class (ii) rho^-s. The size of the repeat and GC content are important in favouring illegitimate site-specific recombination events at GC clusters rather than in the AT spacers. That is the higher stability of the heteroduplex formed at recombination seems to be the main reason for this preference.

V. Summary

This thesis has focussed on the process of mitochondrial transcription and RNA processing. It can be divided into three phases:

1: Construction of a fine restriction enzyme site map of the 5' end of the gene for apocytochrome b (cob) and the region upstream of it, which includes the gene for the glutamic tRNA ($tRNA_{glu}$), has lead to the detection of a previously uncharacterised strain polymorphism which lies between the gene for $tRNA_{glu}$ and its promoter. The additional sequences are an almost perfect direct repeat of the adjacent sequences.

2: A detailed transcript analysis of the genome segment encompassing cob and the $tRNA_{glu}$ gene has been carried out. The approach taken involved a comprehensive investigation of the transcription products of a single rho^- genome. This represented the first such detailed analysis and has revealed information of uppermost interest and relevance for an understanding of mitochondrial transcription:

- The detection of transcripts several times longer than the repeat unit of the rho^- indicates that the rho^- repeat units are transcribed sequentially in a continuous manner. By

extension to the wild type situation this indicates that transcription units are defined by promoters at their 5' end and processing sites at their 3' end.

- Several putative precursor-tRNA transcripts have been detected which fit into a processing scheme involving at least 3 different trimming events.

- The enrichment of such pre-tRNA transcripts in cob intron mit$^-$ strains indicates an ,as yet, uncharacterised interaction between intron processing and tRNA processing.

- Analysis of rho$^-$ transcripts demonstrates that removal of certain mitochondrial introns takes place in the absence of mitochondrial proteins.

3. The third phase of this dissertation involved the characterisation of a mit$^-$ deletion which revealed several points of interest, relevant to an understanding of the transcription of this region.

-DNA sequence analysis has allowed a precise localisation of the excision sites of the deletion to points 70 bps upstream of the cob initiator codon and 10 bps upstream of the 5' end of the cob mRNA.

-Transcript analysis of the mit$^-$ strain supports the possibilty that the 5' terminus of the cob mRNA represents a processing site of $tRNA_{glu}$- cob co-transcript and that

$tRNA_{glu}$ and cob are transcribed from the same promoter.

-Deletion of the cob 5' processing site leads to rapid degradation of transcripts downstream of the cob leader. This indicates that correct 5' processing is essential for the production of stable gene transcripts.

-Examination of the DNA sequences that define the deletion reveal that the excision site is defined by long direct and inverted repeats probably involved in the deletion event. Taken together with the sequence data for the strain polymorphism (see above) this indicates that 'slip-mispairing' (Albertini et al., 1982) is involved in mitochondrial deletion formation.

VI. Prospects

This thesis has shed a great deal of light on the processes involved in mitochondrial transcription and processing. It would be of considerable interest to define the nucleotide sequences involved in the excision events which produce the transcripts detected in this thesis. Such experiments are infact underway: use is being made of the technique of S1 mapping whereby ^{32}P end labelled DNA fragments are used to locate the termini of complementary RNA molecules (Berk and Sharp, 1977). Furthermore, this dissertation represents a starting point for several new projects in this lab. A point which will no doubt be pursued in

this lab is to characterise more precisely the factors which are involved in transcript processing and transcript stability. One could envisage two phases: the first would involve the induction and isolation of mutants (mitochondrial or nuclear) affected in RNA processing. It would be possible to use the mit^- deletion M9410 as screen for the isolation of further mit^- strains localised within the region encompassed by the M9410 deletion. Testing for such strains would be rather troublesome as it would involve a form of negative testing. that is, the selection of those strains that do not recombine with M9410 to produce respiratory competent cells. Another approach would be to attempt to isolate nuclear or mitochondrial revertants of M9410. One could envisage that nuclear revertants would include strains possessing alterations in the genes coding for certain processing enzymes such that correct processing of the cob M9410 mRNA would be restored. The second phase would include a precise physical characterisation of the transcripts produced by these revertants, which would be accompanied by attempts to isolate the nuclear gene involved in the reversion event. To this end, use could be made of a yeast gene clone bank to identify the gene concerned.

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